EFFLUX OF NOREPINEPHRINE FROM PLATELETS IN RELATION TO HYPERTENSION

A clinical and experimental study
on granular exocytosis
and the influence of sodium.

by

INGRID MATTIASSON

Malmö 1983

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Abstract

The efflux of isotope labelled norepinephrine (NE) from preloaded platelets has been studied. The efflux of NE was parallel to the efflux of beta-thromboglobulin, indicating exocytosis of alpha granules. Cell lysis or platelet aggregation could be excluded as the cause of NE release. Thrombin induced an immediate short-term release of NE, but the rate constant was thereafter unchanged. Prostacyclin and prostaglandin E_1 decreased the efflux temporarily, but acetyl salicylic acid in vivo and in vitro did not influence on the efflux rate constant.

The rate constant for NE efflux (k) correlated significantly to the level of the diastolic blood pressure in middle-aged individuals ($r=0.75$ $p<0.001$). NE uptake did not correlate either to the k value or the blood pressure level. 27% of a group of young, normotensive individuals with a family history of essential hypertension had efflux rate constants above those of all the controls, comprised of young individuals with no inheritance of hypertension. This discrepancy could not be ascribed to differences in the blood pressure level.
The rate constant increased when the concentration of Na^+ increased in the incubation medium. The mean k value was higher in a group of rats treated with DOCA-injections and that were given 1% NaCl solution as drinking fluid . As earlier studies have demonstrated a decreased storage capacity for NE in highly sympathetic innervated tissue in DOCA- and salt-treated rats, this latter finding gives an indirect evidence for a parallelity between platelets and neuronal cells concerning NE release. A significant correlation was demonstrated between efflux of NE from platelets and synaptosomes in rats ($r=0.67$ $p<0.001$) as well as between platelets and heart tissue.

Key words

Platelets, norepinephrine, alpha-granules, essential hypertension, beta-thromboglobulin, prostaglandins, sodium, DOCA, acetyl salicylic acid, synaptosomes, exocytosis, sympathetic nervous system, blood pressure.

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III Mattiasson I., Christensson B., Husberg, B. Net efflux rate of norepinephrine from platelets in DOCA- and salt-treated rats. Life Sci 28: 2799-2804, 1981.

IV Stavenow L., Mattiasson I., Theodorsson, B., Hood B. Spontaneous efflux of platelet α-granule components in relation to ^{14}C-norepinephrine in buffers. Life Sci 30: 1899-1905, 1982.

V Mattiasson I., Israelsson B., Stavenow L., Hood B. Effects of thrombin, acetyl salicylic acid and prostaglandins on spontaneous efflux of norepinephrine and beta-thromboglobulin from platelets in calcium/magnesium deficient environment. Accepted for publication in Thrombos Res.

VI Öhlin H., Mattiasson I., Nyström I., Christensson B., Hood B. Efflux of norepinephrine from rat platelets, synaptosomes and heart tissue. Submitted for publication.

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To
Gustav, Gerda and Emma

ABBREVIATIONS

bTG = beta-thromboglobulin

cAMP = cyclic adenonsine monophosphate

DBP = diastolic blood pressure

DOCA = deoxycorticosterone

GSA = growth stimulating activity

LAPF 4 = low affinity platelet factor 4

MAO = monoamine oxidase

NE = norepinephrine

^{14}C-NE = ^{14}C-norepinephrine

PDGF = platelet derived growth factor

PF 4 = platelet factor 4

PGE_1 = prostaglandin E_1

PRP = platelet rich plasma

SHR = spontaneously hypertensive rat

spSHR = stroke prone spontaneously hypertensive rat

Tx A_2 = thromboxane A_2

WKR = Wistar Kyoto rats

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BACKGROUND

The sympathetic nervous system in hypertension.

The role of the sympathetic nervous system in the etiology or the maintenance of hypertension has been extensively studied and debated since the 1950s. Early studies failed to unveil any apparent abnormality of the sympathetic system in the majority of hypertensive patients, mainly because of the use of inprecise or improper means of investigation. After the development of enzymatic isotope-derivate techniques, reliable measurements of plasma norepinephrine (NE) and epinephrine were obtainable and were regarded as an index of sympathetic nervous activity. It was shown that electrical stimulation of sympathetic nerves increased plasma levels of NE (1) and that plasma NE correlated with sympathetic muscle nerve activity, recorded directly from nerve branches in normotensive individuals (2). It was suggested that overflow of transmitter from sympathetic terminals in muscles contributed significantly to the plasma levels of NE at rest. However, no relationship between muscle sympathetic activity and blood pressure level seems to exist, either in normotensive or in hypertensive subjects (3). There is no hemodynamic evidence of increased peripheral resistence in the muscle vessel bed in established hypertension. There is vasodilation in the defense alarm reaction in the muscular regions while there is constriction in splanchnicus. (4,5). Furthermore, it is obvious that even under standardized conditions, plasma levels of NE vary markedly between subjects (6) and that they increase with age (2,7). NE levels are also influenced by mental activity (8). Apart from release of NE related to sympathetic activity, reuptake into nerve endings and catabolism of NE will also influence plasma NE concentrations. These facts may explain the contradictory results in different investigations, some demonstrating elevated levels of plasma NE in hypertension (even when age was corrected for) (9), and others not (10).

These "human-subject" related difficulties have made animal studies favourable, but the experimental results are difficult to relate back to human essential hypertension. However, in animal studies, with the help of labeled compounds and highly specific drugs, it is possible to directly measure the turnover rate, the synthesis rate, the reuptake and the metabolism of neurotransmitters in various organs. In the 1960s a series of studies were made by de Champlain, Axelrod et al (11-13) concerning the relationship between the sympathetic nervous system and the variation of blood pressure induced by

various degrees of sodium intake, ranging from sodium depletion to sodium loading, in combination with deoxycorticosterone (DOCA). Those studies established that, in the rat, sodium balance influenced the activity of the nerve terminals and adrenal medulla, and that those changes may be responsible for the variations in blood pressure under the chosen experimental conditions. In DOCA-hypertensive rats the amount of tritiated NE retained in several organs containing an important vascular innervation was markedly reduced one hour after intravenous injection. The reduction was proportional to the reduction of endogenous levels of NE in the same tissues. The neuronal uptake, as well as the uptake into the storage vesicles, was found to be normal, but the amount remaining in the organelle fraction after four hours was reduced by 45% in the hypertensive animals vs 8% in the normotensive animals. This suggests to the authors that a persistent disappearance of the labelled amine from its storage sites occurs. An alternative explanation could be that the storage sites, i.e. nerve terminal granules themselves had a more rapid turnover. The increased NE turnover was associated with an increase in catecholamine synthesis (14). Studies on the intraneuronal distribution of NE revealed that the fraction of soluble and free NE was consistently greater in the heart of hypertensive rats at various times after administration of tritiated NE, thus suggesting that greater amounts of physiologically active NE were accessible to receptor sites in this condition (12). Serum catecholamine levels were found to be higher in the hypertensive rats (9,15). The increased turnover rate seemed to precede the rise in blood pressure (13,16). A highly significant inverse correlation could be established between the level of the systolic blood pressure and cardiac NE retention (or content) in normotensive and hypertensive rats (17,18). The relationship was significant, even in the hearts of animals within the normal range of blood pressure, suggesting that the level of blood pressure is closely related to the activity of the sympathetic nervous system, even under normal conditions. Chemical sympathectomy by 6-OH-dopamine resulted in a greater lowering of blood pressure in DOCA- and sodium-hypertensive animals (19).

The Japanese strain of spontaneously hypertensive rats (SHR) has, since the early 1970s been a frequently used model for studies on hypertensive pathogenesis. In this strain direct recording of the electrical activity of the splanchnic nerve revealed that the peripheral sympathetic tone was markedly increased in the hypertensive animals and that the NE turnover rate was increased in the hearts of young SHR (20-22). Contradictory results have also

been reported, which probably could be ascribed to the use of inadequate controls (19,23). In the stroke prone SHR (spSHR) neonatal sympathectomy with 6-OH-dopamine blunted the rise in blood pressure normally seen until reinervation was established (24). Salt loading caused a distinct increase in plasma NE concentration in this strain, potentiating an already elevated sympathetic activity (25).
NE release was significantly accelerated in spSHR by depolarization with a low potassium ion concentration (26). Responses evoked by stimulation of the renal sympathetic nerves were greater in young SHR (7 weeks) than in controls, which was ascribed to a larger release of NE from the nerve endings (27).

The release of norepinephrine from nerve cells

The accelerated release of NE from neuronal cells may be induced by indirectly acting sympathomimetic amines or other drugs, or by the depolarization which is normally caused by the arrival of propogated nerve impulses to the nerve terminals. Under resting conditions there is a spontaneous "leakage" of NE (28).

The bulk of the evidence concerning transmitter release is biochemical. However, fractionation of the particulate fraction of nerve cells by centrifugation has shown that at least two types of particles can store NE (29) and that these particles correspond to the large and small dense-cored vesicles identified by electron microscopy (30). Proteins which are associated with the latter vesicles are dopamine-β-hydroxylase (soluble and membrane bound), chromogranin A and other chromogranins. In the small vesicles, dopamine-β-hydroxylase activity has been measured (31). There is a secretion of these high molecular weight proteins from sympathetic nerves upon nerve stimulation. As none of the membrane bound constituents of the vesicles are released, nor the proteins from the cytosol, the most likely way in which this secretion can take place is by exocytosis. All other soluble constituents of the vesicles, including NE, must then also be released in the same way (31).

It is generally accepted that an increase in the intracellular Ca^{2+} concentration is the signal which initiates secretion of neuron transmitter substances, although the precise mechanism of calcium's action has not been elucidated. Based primarily on studies by Katz et al, (for review see ref 32) the

"calcium hypothesis" was formed. These workers proposed that depolarization causes an increase in Ca^{2+} permeability of the pre-synaptic terminal plasmalemma. Ca^{2+} enters the terminal, moves down its electrochemical gradient and leads to an increase in intracellular Ca^{2+} concentration. This induces fusion of synaptic vesicle membrane with the plasma membrane so that the vesicular contents are extruded into the synaptic cleft. A lot of hypotheses have been presented to explain Ca^{2+}-dependent exocytosis. It has been proposed that Ca^{2+} binds to the vesicle membrane and intracellular surface of the plasmalemma, thereby neutralizing the negative surface charges on these membranes and thus reducing the electrostatic repulsion. Other hypotheses involve Ca^{2+}-activated systems, which pull the vesicles up to the plasmalemma and/or displace the polar groups on the surfaces of the vesicles and plasmalemma (for review see ref 32).

Ca^{2+} is transported out of the neuron against a very large electrochemical gradient. The ratio between extra- and intracellular Ca^{2+} is in the order of 10^{-4}, requiring some type of energy-linked transport system. Extrusion of Ca^{2+} requires the presence of Na^{+} in the external medium (33). An increase in intracellular Na^{+} and/or a decrease in extracellular Na^{+} tends to promote Ca^{2+} entry into the terminal (34). These observations suggest that Ca^{2+} extrusion from the nerve terminals may be mediated by a Na^{+}-Ca^{+}-counter transport system powered by energy from the Na^{+}-electrochemical gradient.

Platelet morphology

Although platelets are but fragments of megacaryocyte cytoplasm, they have evolved a complex machinery to subserve a lot of functions in hemostasis and tissue repair. Newly formed platelets often contain ribosomes and RNA from the parent cytoplasm, but in general platelets are incapable of synthesising proteins in biologically significant quantities (35). Examination of the mature platelet demonstrates the presence of a canalicular system, the dense tubular system, through which plasma borne substances may enter the platelet for transport to the platelet granules (36). Widely scattered throughout platelet cytoplasm are the microfilaments, composed of actin and myosin, which have contractile properties (37). The microtubules are arranged in the equatorial plane of the platelet as a well ordered concentric ring lying just beneath the plasma membrane. Following receipt of a hemostatic signal at the surface membrane, the ring of microtubules reduces in diameter, contracting towards the center of the cell, thereby concentrating the granular organelles within a central cytoplasmic zone. At the same time platelets are transformed

from the discoid form to a spherical shape and pseudopodea arise from the surface. This process is called the "shape change", and also involves the contraction of the submembraneous network of actomysin filaments by the polymerisation of platelet actin and myosin, a Ca^{2+}-dependent process (38,39). (For a detailed review of platelet morphology see ref 40). Electron microscopy reveal the presence of at least three types of organelles in the platelet. Two of these are morphologically distinct granules, the dense bodies and the alpha-granules, and the third is a population of vesicles containing lysosomal enzymes. However, by secretion experiments, subcellular fractionation and from studies on platelets from patients with storage pool deficiencies, it has been proposed that at least four kinds of storage organelles exist in platelets (41).

The dense bodies, or dense granules, are electrone dense, osmophilic and have a high specific weight, giving them the highest density of any platelet organelle. They contain serotonin, Ca^{2+}, ATP, ADP and pyrophosphate. The mechanism for storing serotonin is not completely clear. It has been proposed that serotonin is incorporated into micelles together with ATP. Whether Ca^{2+} is necessary for the process or not is debatable (42). About 60-70% of platelet Ca^{2+} is stored in the dense bodies, but is not exchangeable with either cytoplasmic Ca^{2+} or extracellular Ca^{2+} (39). About 65% of the total content of adenine nucleotides is stored in the dense bodies as well, exchanging only slowly with the metabolic pool (42).

Alpha-granules: The main constituent of the alpha-granules are platelet specific proteins, cationic proteins and coagulation factors. Their localization in alpha-granules has been demonstrated by subcellular fractionation (43,44). No information about their storage mechanisms is yet available and it is possible that alpha-granules are heterogenous in their capacity for storing different proteins.

The platelet specific proteins whose presence in circulating blood is regarded to be a measure of platelet activation in vivo are beta-thromboglobulin (bTG) and platelet factor 4 (PF4). The cationic proteins that have as yet been identified are the platelet derived growth factor (PDGF), fibronectin, the permeability factor (which increases permeability through inducing histamine release from mast cells), the chemotactic factor from polymorphonuclear leukocytes, the bacteriocidal factor, and the platelet mitogenic factor (45,46). The coagulation factors are fibrinogen, Factor Va (42) and factor VIII

related antigen (45).
bTG has a molecular weight of 35,800 (47) and is present in a high concentration in the alpha-granules (40-60 ug/10^9 platelets = 30-40% of granule proteins) (48). Its amino acid sequence is very close to that of PF 4 (49). bTG appears to be a proteolytic product of low affinity platelet factor 4 (LAPF 4), resulting from cleavage of a tetrapeptide from the N-terminal end of each subunit of LAPF 4. At the present time, LAPF 4 and bTG cannot be distinguished immunologically (50). The function of bTG is unknown, but in one report it has been demonstrated to reduce the production of prostacyclin-like activity in cultured bovine endothelial cells, which are shown to possess a specific receptor for bTG. The characteristics of that receptor suggested that bTG may act locally at high concentrations to favour platelet aggregation by diminishing prostacyclin production (51). PF 4 did not compete for the receptor and had no similar biological effect.
PDGF is composed of two different polypeptide chains covalently linked to make up a protein with a molecular weight of slightly more than 33,000 (52). It has been proposed to be the principal mitogen responsible for the induction of DNA-synthesis in fibroblasts, myocytes and glia cells and it acts by initiating cell cycle traverse and requires the presence of plasma constituents for a maximal growth response to occur (53). Recently PDGF has been demonstrated to stimulate prostacyclin synthesis in physiological concentrations (54). It also stimulates LDL-receptor activity in cultured human fibroblasts (55).

The third and fourth group of organelles are lysosomal in nature. The lysosomal enzymes are secreted by platelets treated with thrombin or collagen but not with ADP or adrenaline. Their broad distribution on a density gradient indicates that the lysosomes are more heterogenous than the above mentioned populations of granules. The criterion upon which these organelles are divided into two groups is based on their secretion behaviour (41).

Platelet adhesion and aggregation.
Under normal circumstances circulating platelets are nonadherent to other blood elements and intact endothelium. Vascular disruption exposes blood to subendothelial connective tissues and initiates the platelet reactions of adhesion, aggregation and release, leading to the formation of a hemostatic plug. The process of platelet adhesion involves the interaction of platelet surface glucoproteins with connective tissue elements of the subendothelium and requires the factor VIII related antigen as a plasma cofactor (56).

Adherent platelets release their dense granule and alpha-granule contents. Thrombin is generated locally in small amounts and thromboxane A_2 (TxA_2) is synthesised from arachidonic acid liberated by membrane phospholipases. Released ADP, TxA_2 and thrombin recruit additional circulating platelets to the enlarging platelet mass. Thrombin generated fibrin stabilises the platelet mass. Prostacyclin released by the vessel wall in response to thrombin limits formation of thrombin by inhibiting further platelet aggregation (45). Platelet agonists of physiological significance are thrombin, epinephrine and collagen. Most of these agents appear to act by binding to specific receptors on the platelet surface membrane. (For a detailed review of platelet adhesion and aggregation see ref 57). Platelet aggregation will not occur in the absence of either extracellular divalent cations or fibrinogen.

Platelet release reaction.

The release reaction is a secretory process whereby substances stored in platelet granules are extruded from the platelets by exocytosis. Alpha-granule contents are more readily released than the dense bodies, and acid hydrolase-containing lysosomal granules are released only with high doses of collagen or thrombin (43,58). The exact mechanisms by which the agonists initiate granule release are unclear. Like platelet adhesion and aggregation the release reaction is a membrane mediated process involving specific glucoproteins located on the platelet surface. Although thrombin and collagen can induce platelet aggregation and release directly, these agents also activate the platelet arachidonic acid pathway - probably through activation of membrane phospholipase. Liberated arachidonic acid is rapidly converted to TxA_2, which then mobilizes Ca^{2+} from various intracellular storage sites. Mobilized Ca^{2+} is probably the final mediator of platelet aggregation and release (59,60). Elevated cytoplasmic Ca^{2+} complexed with calmodulin may then activate kinases producing phosphorylation of platelet myosin, triggering contraction and initiating secretion (61,62). Elevation of free Ca^{2+} also activates the Ca^{2+}-sensitive phospholipases, thereby further amplifying the process (45). External Ca^{2+} plays, at best, a minor role in the events which trigger platelet activities since the influx of Ca^{2+} through the plasma membrane coincides with the release reaction (63).

Platelets as a model for neurons.

The concept of platelets as a model for monoaminergic neurons is based on the presence, in platelets, of an amine system consisting mainly of serotonin,

but also of other amines. This system has similarities to that found in nerve tissues, especially with regards to uptake, storage, release and metabolism of amines.

No biosynthesis of monoamine seems to be possible in the platelets since neither tryptophan hydroxylase nor dopamine-β-hydroxylase have been found in platelets. Platelets probably take up most or all their serotonin after they have been released from the megacaryocytes, which contain very little serotonin. The principle source of the platelet serotonin is thought to be the enterochromaffin cells. The biogenic amines enter the platelet by passive diffusion. This uptake shows linear dependence on the external amine concentration. For serotonin there exists a second uptake process, which has a high affinity for serotonin, is saturable and depends on Na^+ and on metabolic energy. This carrier mediated, specific uptake is much more efficient than the passive diffusion when concentrations of the amine in the medium are low. The specific uptake mechanism for serotonin seems to be located at the plasma membrane. Active uptake of serotonin by platelets is inhibited by numerous drugs which also interfere with amine uptake in neuronal tissue, for example tricyclic antidepressants. (64).

The uptake of dopamine and NE in platelets is known to be much less pronounced than that of serotonin. The K_m values for the dopamine and NE uptake in neuronal tissue were 2-3 orders of magnitude lower than in platelets. In contrast the K_m for serotonin is of the same order in both neurons and platelets. NE uptake is a saturable process which depends on Na^+; the process is impaired at low temperature and by metabolic inhibitors. Various biogenic amines, like serotonin, tryptamine and tyramine, as well as drugs like desmethylimipramine, amphetamine, adrenergic neuron blockers (e.g. guanethidine, alpha- and beta-blockers), and antihistamines interfere with NE uptake. This indicates that an energy dependent carrier mediated mechanism is involved in the uptake of NE by human platelets, but that the passive component of NE uptake is dominant (65,66).

At the membrane of adrenal chromaffine granules there is probably a proton pump, driven by Mg^{2+}-ATP-ase which creates an electrical and/or chemical gradient responsible for the catecholamine uptake. Evidence for such a mechanism also exists at the membrane of serotonin granules (67). The accumulation of serotonin in the subcellular serotonin organelles occurs by a mechanism different from that responsible for the uptake of the amine at the

level of cytoplasmic membrane. This difference is most evident in inhibitor experiments. Thus, reserpine, which does not affect the serotonin uptake at the cytoplasmic membrane level, markedly inhibits serotonin uptake into the isolated serotonin storage organelles. On the other hand, imipramine impairs the serotonin uptake across the cytoplasmic membrane but does not markedly affect the accumulation of the amine in the storage organelles (68). Although the contents of other endogenous amines in platelets are, in general, much lower than that of serotonin, the biogenic amines accumulate in their highest concentrations by far in the granular fraction of subcellular fractionation experiments (65). In isolated organelles of rabbit platelets serotonin is taken up most effectively (100%), followed by dopamine (68%), epinephrine (36%), NE (29%) and histamine (2%) (69). Studies on the endogenous concentrations of monoamines show that the platelet content of free catecholamines is about a thousand times lower than that of serotonin, NE being the prevailing catecholamine. The ratio whole platelet/plasma for the free NE is almost 500, for sulphoconjugated about 150. The ratio of total monoamine content organelles/platelets is about 220 for serotonin and 130 for norepinephrine (70).

In both platelets and neurons three main types of release mechanisms can be distinguished: by exocytosis, by reserpine and by phenyl - or indolyl - alkylamines. The mechanisms underlying these three types of release seem to be similar in platelets and neurons, although the stimuli for exocytosis differ in both cell types. In platelets release experiments have been mainly focused on serotonin, whereas in neurons both serotonin and catecholamines are the center of interest. That neurons release NE by exocytosis during nervous stimulation has been shown by studying the parallel release of intragranular proteins. This Ca^{2+}-dependent release of vesicular contents by exocytosis has been demonstrated in many exocrine and endocrine glands as well as neurosecretory organs. The convergent observations lead to the conclusion that all of these secretory processes are fundamentally similar. Thus, information about the secretory process obtained from any one type of preparation may provide useful insights into the mechanisms of secretion in other secretory systems as well (32).

Recently it has been demonstrated that the serotonin induced shape change reaction in platelets is mediated by the stimulation of a specific serotonin receptor, different from the receptor for serotonin transport, that reacts to the same serotonin antagonists as the neuronal serotonin receptors of some areas of the CNS. It has also been demonstrated that the shape change reac-

tion is a sensitive mean of detecting those basic proteins and polypeptides which cause functional change in neuronal cells (71).

The possibility has to be considered that in certain neuropsychiatric disorders some general defect in the uptake, metabolism, storage and/or release of monoamine may exist. Such a disturbance might also be manifest in platelets, provided they resemble the nervous system with regard to their monoamine system. Platelets of patients with Parkinson's syndrom, which is biochemically characterized by a loss of dopamine in the nigrostriatal brain centers, have been found to take up less ^{14}C-dopamine than aged matched controls (72). A decreased storage of dopamine has also been proposed (73). The role of platelet serotonin has perhaps been investigated most thoroughly in Down's syndrome, where a reduced content of serotonin has been solidly documented and repeatedly confirmed. The reason for this decreased platelet serotonin content has been debated. One study capable of explaining most of the known biochemical abnormalities in Down's syndrome platelets was made by McCoy et al and demonstrated a decreased Na^+/K^+-ATPase activity and an increased Na^+ content intracellularly (74). The serotonin uptake has been reported to be greatly diminshed in platelets from patients with endogenic depression (75,76). (For review see ref 77).

The B-form of monoamine oxidase (MAO) is present in human platelets and seem to be under strong genetic control (for review see ref 78). MAO activity in the brain as well as in platelets has been found to be low in patients with alcoholism, suicidal behaviour and cycloid psychosis (79-84).

THE PRESENT STUDY

Design of the study

Earlier studies refered to in the previous section give evidence for a parallel behaviour between neuronal cells and platelets with respect to storage and release of catecholamine. Data from experimental animal hypertension studies propose an interference between the sympathetic nervous system and sodium, inducing a decreased storage of NE during hypertension. These data made us start an investigation concerning the possible relationship between the blood pressure level and the handling of NE in platelets in middle-aged individuals (paper I).
As there seemed to be a correlation between these parameters, the study was designed to try to answer the following questions:

1. Could the increased efflux of NE from platelets be already registered before the blood pressure starts to rise or is it secondary to the blood pressure rise in itself? (paper II)

2. Does sodium interfere with the storage and handling of NE in platelets? (paper I and III)

3. Is the increased efflux of NE a function of a selective release of NE from intragranular or cytosolic binders, or is it an effect of increasing exocytosis of whole granules? (paper IV)

4. Is the NE release influenced by the addition of substances which increase or decrease platelet aggregation or adhesion? (paper V)

5. Are there any parallels between neuronal tissue and platelets with respect to NE release? (paper III and VI)

METHODS

Norepinephrine efflux.

The method for determining of NE efflux is described in detail in papers I and VI. EDTA was used as anticoagulant. PRP was incubated for 90 min with isotope labeled NE. The tubes were incubated at 37°C with continuous shaking in an atmosphere of 95% O_2/5% CO_2. Platelets were separated by centrifugation and resuspended in Krebs-Ringer bicarbonate or Hepes buffer without Ca^{2+} and Mg^{2+} and were again placed in a water bath at 37°C with continuous slow shaking. Aliquots were taken for the determination of uptake at start of the efflux study and for released NE, at continuous time intervals. In the first study (paper I) efflux was studied for a duration of 120 min and in paper II-IV for 60 min, with a 80 min value was a control value. In paper VI the efflux was studied for 20 min to enable comparison with the efflux from neuronal tissue.

The increase in radioactivity in the supernatant after platelets had been separated, compared to the start value (zero time value) was taken as an expression of the NE released from the platelets. If these values were plotted against time on a semilogarithmic diagram a straight line was obtained during the initial phase of efflux. The faster the efflux, the earlier a slowing down of the efflux rate occurred. The regression coefficient (k) for the linear part of the curve was used as an expression of the initial efflux rate. A plateau in the efflux curve was defined as three successive values varying <10%.

In the first investigation (paper I) platelets were also incubated with ^{3}H-serotonin as well as ^{14}C-NE in the same sample.

We are aware that during the incubation period NE may be partly metabolized and that the measurement of radioactivity during efflux phase will include both NE and metabolites. As we do not think that this would affect the main lines of arguments, we have chosen to regard the labeled efflux as identical with NE. By using logarithmic curves the influence of platelet count and variation in platelet loading was eliminated. This favours the comparison of efflux curves between individuals. It also potentiates the initial efflux phase, which is less influenced by external factors. However, it was obvious that the efflux curves were only initially linear in a logarithmic plot and

that there was a continuous flattening of the curve. What is measured is the net efflux - the difference between efflux and uptake. Later in the process the uptake will be larger. The amount of NE available for the platelets during the efflux study is 50-100 times below the amount added during the uptake phase. Washing of the platelets was avoided after centrifugation in order to minimize the loss of platelets and the risk of damage of the cells during too many centrifugation processes. This gave a background activity of about 1000 CPM, which was subtracted from all efflux values. Variations in the background from 500-2,500 CPM, which was tested by adding ^{14}C-NE to aliquots at start of efflux study, did not influence on the k-value. During the 60-120 min period during which efflux was studied in these clinical investigations reuptake seemed to have little influence on the k-values. This was verified by testing the influence of desmethylimipramine at a concentration of 10^{-5}mol/l, which inhibits uptake by 65%. No influence on the k-value was seen in four experiments. Variations in incubation time between 30 and 180 min did not influence on the k-value, nor did variations in the amount of isotope added up to three times higher than the concentrations used in the clinical experiments (unpublished data).

During the period in which the study on relatives of hypertensive individuals (paper II) was made, k-values were determined in an individual not included in the study ten times during a seven month time period. The mean k was 15.3 ± 3.0 min^{-1}.

Parallel studies of lactate dehydrogenase release and NE release seem to exclude the possibility of cell damage as an explanation of the spontaneous NE release. The amount of lactate dehydrogenase released was 0-9% of the total lactate dehydrogenase content and did not parallel the NE release (paper IV).

Recently the possibility for starting analyses of intracellular concentrations of Na, K, Mg and Ca became available. Measurements on these ions have been made during all phases of the handling of the platelets. These analyses have revealed that intracellular content of Na rose rapidly when the samples were placed in ice water but also thereafter (fig 1). Intracellular contents of K decreased in the same way. Very small differences were registered in Ca and Mg however, even at the end of the 80 min efflux study. If blood was sampled with the addition of EGTA, the differences in K and Na were much less and the increase in Na and decrease in K was reversed if Mg^{2+} was added to the buffer.

This suggests that the Na/K-ATPase function is at least partly inhibited in the presence of EDTA. However, the k-value for NE was the same with both sampling techniques (unpublished data).

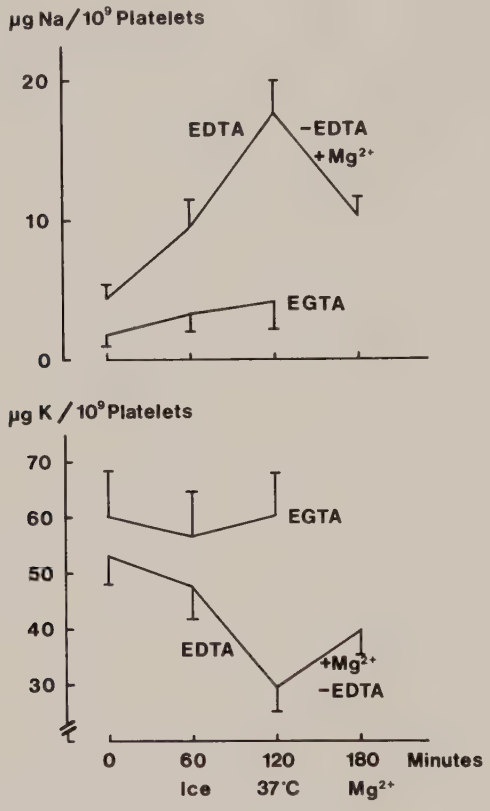

Fig 1.

Intracellular content of Na and K in platelets during different phases of the uptake and efflux study. Blood from an individual was sampled with addition of EDTA or EGTA (0 min). The tubes were placed in ice water for 60 min. After preparation of platelet rich plasma this was placed in 37°C for another 60 min. After centrifugation platelets were transferred to the HEPES buffer (see paper VI) with addition of 1 mM Mg^{2+}. Determination of intracellular Na and K was made after 60 min in this medium.

Beta-thromboglobulin

bTG was determined with a radio immuno assay technique described in detail in paper IV.

Growth stimulating activity

GSA was quantified by its ability to stimulate the incorporation of ^{3}H-thymidine in rabbit aortic smooth muscle cells. The technique is described in detail in paper IV. The growth stimulating activity probably mostly contained PDGF, but the contribution of other growth stimulating substances can not be excluded.

Preparation of synaptosomes.

Synaptosomes were prepared according to a method described by Cotman (85). The technique is described in detail in paper VI.

RESULTS AND DISCUSSION

The relationship between NE efflux and blood pressure (I).

The uptake and efflux rate coefficient (k) was studied in 63 middle-aged individuals, 48 men and 15 women, with a range of diastolic blood pressure (DBP) between 70 and 150 mm Hg. A close correlation was demonstrated between k and DBP ($r=0.75$ $p<0.001$). No correlation was seen between uptake and DBP, nor between uptake and k. Later was verified in aliquots with varying ^{14}C-NE that k is not influenced by the uptake values when calculated in a semilogarithmic way.

There was a great intra-individual variation in uptake values in spite of very standardized loading procedures, which we today have no explanation for.

NE efflux in normotensive relatives of hypertensive individuals (II)

Early was recognized that essential hypertension is influenced by genetic factors (86-89). With one or two hypertensive parents, the risk of hypertension is 9-15 times greater in offspring than when none of the parents are hypertensive (88). A study made at this clinic gave a prevalence of hypertension or hypertensive disease of about 50% when studying the blood pressure of 1,075 relatives to 106 probands with treated hypertension and having at least one earlier known relative with hypertension (90). A selected group of individuals from families with a heavy history of essential hypertension should then contain about 50% of individuals who have genetic disposition for developing essential hypertension. We wanted to study the efflux of NE in a group of normotensive young individuals from such families to find out whether or not the increased NE efflux could be demonstrated in some of these - i.e. if the increased efflux develops before the blood pressure starts to rise.

The individuals were taken from the earlier study, made two years before by Ohlson et al and described elsewhere in detail (90). By investigation of consecutive records of hospitalized or out-patient records during four years, 106 investigated and treated patients were selected as probands. All subjects entering the study should have at least one close family member with hypertension and/or cerebrovascular disease with onset before the age of 60. Patients with manifest diabetes or secondary hypertension were excluded. All mature relatives, up to 60 years of age without verified treated hypertension, and living in Malmö or its surroundings (n=339) were invited for examination.

For the present study, individuals were invited who had hypertension in the family for at least two generations. They were first degree relatives to an individual with essential hypertension which was verified by examination. They should have DBP $\leq$90 mm Hg at the first examination and be between the ages of 18-40 years. For practical purposes, those living too far from Malmö were excluded. All individuals taking drugs of any kind, including contraceptive pills, were also excluded. In this way a group was obtained comprised of 44 individuals, 31 males and 13 females. The lower number of females was partly ascribed to the fact that many had to be excluded because of their use of contraceptive drugs. The control group was comprised of 31 individuals (m=21, f=10) between 22 and 40 years of age. These were mainly colleagues and hospital workers. The controls were all definite in having no hypertension or cerebrovascular disease in the family (parents, grandparents and siblings). In all cases where there was any doubt about these data, the individual was not accepted as a control.
A third group was comprised of 18 relatives (m=10 fe=8) above 40 years of age with the same criterion of inheritance as the younger group. Blood pressure should be $\leq$95 mm Hg.

This study demonstrated that the mean k-value was higher in the young relatives and that 27.5% of them had higher k-values than any of the controls. The mean DBP was somewhat higher in the young relatives, but by matching relatives and controls according to DBP, age and sex, it was demonstrated that the difference in k could not be ascribed to the difference in blood pressure. In controls there was a significant correlation between k and DBP ($r=0.39$, $p<0.05$), though weaker than in the mixed middle-aged group ($r=0.75$, $p<0.001$) (paper I). This might at least partly be ascribed to the fact that DBP varied only 60-85 mm Hg, compared to 70-150 mm Hg in the mixed group of middle-aged individuals. There was no correlation between DBP and k in the young relatives. DPB range in this group was 60-100 mm Hg.

There was no difference in uptake between the groups as in the mixed group of middle-aged individuals nor between males and females. Nor was there any difference in k between males and females.

Because of the close correlation between k and DBP in the 15-20 year older, normotensive and hypertensive individuals (paper I) one could speculate about the possibility that those individuals having the high k-values might be the future hypertensive group among the relatives. If this was the case, one

would not expect finding high k-values in normotensive, elderly individuals. This was the reason of also including a group of middle-aged normotensive relatives in the study. The 18 individuals included were all those who fulfilled the criterial for entrance in the study although the limit for DBP was raised to ⩽95 mm Hg. Some had to be excluded because of medication (analgetics, neuroleptics) and some had developed hypertension since the first investigation and were under treatment for that. k in this group was significantly lower than in the young relatives, with the peak in k-distribution at the same level as the controls. 2 individuals (11%) had k-values above the controls, 32.8 and 27.4. The age of these was 51 and 42 years respectively and their DBP was 75 and 80 mm Hg. The results from the group of middle-aged individuals support the theory that rapid efflux of NE might indicate an increased risk of developing hypertension, since these selected normotensive individuals from families heavily loaded with hypertension behaved more like the controls having no inheritance of hypertension. In this group of relatives (older than 40 years), one could expect an accumulation of individuals who are not going to become hypertensive, since those already hypertensive have been excluded from the material. It would have been even better to study an even older group of relatives, but the number of available relatives was too small to make any conclusions possible in such a study. The value of high k as a predictor of the risk of developing hypertension can only be validly tested in long term follow up studies. These are currently in progress.

The influence of DOCA and salt treatment on platelet efflux of NE in rats (III).

Since it was demonstrated in the studies made by de Champlain et al (17) that DOCA and/or salt treatment in rats reduced the retained amount of NE in the granular fraction from tissues with a rich sympathetic innervation, we wanted to test if the same treatment had any effect on the NE release from platelets. This was essential in order to evaluate the validity of the platelet as a model of neuronal granular storage. Wistar Kyoto rats were treated with DOCA injections in the same dose as in the investigations referred to above and 1% NaCl was given as drinking fluid for 4 weeks. In de Champlain's study the rats were uninephrectomized, but later investigations have clearly shown that hypertension develops within 4 weeks with these doses of DOCA, even if both kidneys have been preserved (91).

Because of the small amounts of blood available in each rat, PRP had to be

pooled from three animals to obtain the necessary amount of platelets for the efflux study. This fact, together with the earlier documentation of the effects on blood pressure made us abstain from individual blood pressure measurements in the rats. Heart weight was taken as an indirect measure of blood pressure, and was significantly higher in the DOCA-salt treated group. Mean k was also higher in this treated group with little overlaping between the treated group and the controls. These data support the parallel relationship between the platelets and the neurons with respect to NE release. It also demonstrates that interfering with the sodium balance interferes with the platelet storage of NE.

In vitro experiments in human platelets at different Na-concentrations both in the preincubation and the efflux buffer showed that the efflux rate was higher at higher Na^+ concentration in the interval 110-170 mM (paper I). Later, as yet unpublished studies, made when the bTG radio immuno assay had been available, showed that higher Na^+ concentration in the incubation medium also produces an increase in the net efflux of bTG in aliquots (Fig 2).

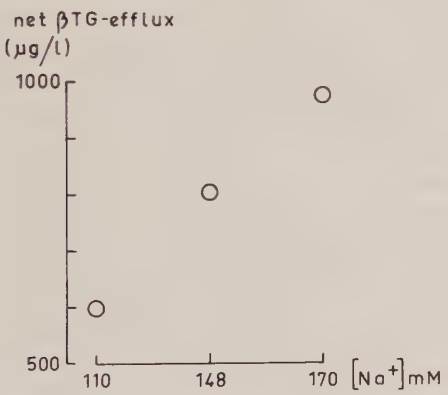

Fig 2.

Net efflux of bTG from aliquots incubated in buffer with varying Na^+ concentration. Efflux was studied during 60 min with the same method as described in paper I.

The relationship between NE and alpha-granule proteins on spontaneous efflux (IV)

In the study on middle-aged individuals, platelets were also incubated with ^{3}H-serotonin (paper I) together with ^{14}C-NE. The release of serotonin was much lower than the release of NE. In earlier studies it was also stated that the spontaneous release of serotonin was very low (92) and later studies confirmed our findings about the different kinetics of serotonin and NE release (93). This finding opens at least four possible explanations. If

serotonin and NE were stored in the same granula, i.e. the dense bodies, they would have to be selectively released from common or separate binding sites with different kinetics. There is also a possibility of a greater pool of extragranular cytoplasmic NE that would be released while the intragranular NE was stored together with serotonin. However, if the earlier reported figures on the accumulation of NE in the granular fraction, with a 120 times higher concentration of NE in the granules, is correct (70), this latter explanation seems less possible if the amount of NE released during 120 min, with a higher velocity than serotonin, is compared to the total amount of NE taken up in the platelets. When efflux was rapid the part of NE released linearly in a logarithmic plot comprised more than 50% of the total amount of accumulated NE.
If reuptake of released serotonin was more efficient than reuptake of NE the resultant in the supernatant would also be a decreased net efflux of serotonin compared to NE. However, inhibition of reuptake had little influence on the spontaneous liberation of both substances and differences in reuptake does not seem to explain the noted difference in net efflux (93).

The fourth explanation would be that NE and serotonin are stored in different granules and with different kinetics. bTG, PF 4 and PDGF are platelet specific alpha-granular proteins. We studied the release of NE, bTG and growth factors during the same experimental conditions as in the clinical studies.

It was demonstrated that the quotient of bTG and NE was fairly constant during 80 min of spontaneous efflux. There was also a continuously increased efflux of GSA. This seems to favour the theory that NE is stored in the alpha-granules, and is released with a kinetics that differs from that of the dense bodies.

Earlier studies with ultracentrifugation of sonicated platelets through a sucrose gradient point to storage of NE in the same fraction as serotonin (70). However, it is now obvious that sonication will give aggregates of organelles instead of isolated granules. This has been ascribed to the existence of myosin on the surface of the granules and actin in the cytoplasm, favouring aggregation (94,95). This aggregation could be avoided by using newer techniques, but with the techniques used in the former separation studies, the possibility of organelle aggregates can not be excluded, thereby minimizing the possibility of obtaining pure alpha-granule and dense body preparations.

Reserpine seems to selectively release dense bodies, but leaves the alpha-granules intact (96). We therefore studied the influence on the NE efflux with the addition of reserpine. No effect was seen on the efflux of NE, although serotonin was released (Fig 3). This finding also supports the conclusion that NE is stored in the alpha-granules (unpublished data).

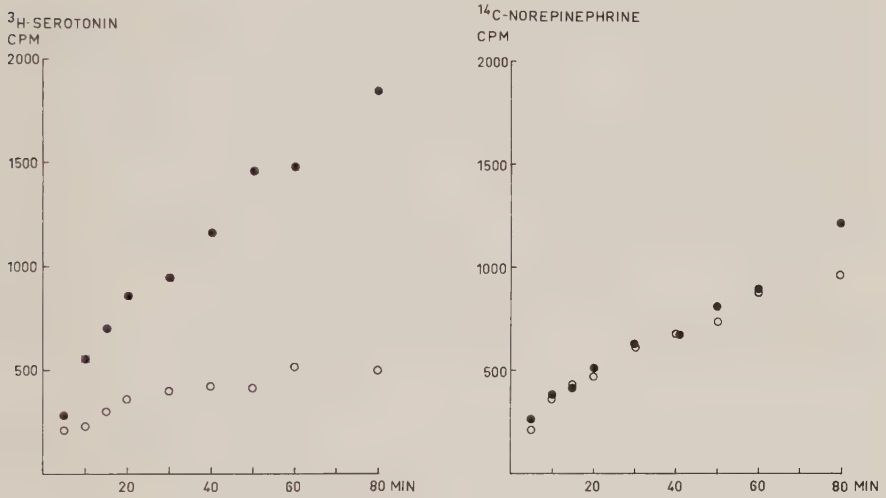

Fig 3.

Four aliquots of platelet rich plasma were prepared. Two were incubated with ^{3}H-serotonin and two with ^{14}C-NE. ● Reserpine (10^{-5}M) was added at start of efflux study. ○ control.

The mechanism responsible for the increased NE efflux thus seems to be an increased alpha-granular exocytosis rate. It is obvious from previous studies that the characteristics of alpha-granular release are different from the release of serotonin and acid hydrolases. Low concentrations of thrombin and collagen gave a more pronounced release of bTG, platelet factor 4 and PDGF than of serotonin (43,44) and high concentrations of thrombin were required for the release of acid hydrolases. Arachidonic acid caused more extensive release of platelet factor 4, bTG and fibrinogen than of serotonin at all

concentrations which induced release (43). The major release of PDGF, PF 4 and bTG occur between 0.015 and 0.025 units of thrombin per ml of gel-filtrated platelets with 70-80% of each of these three components being released by a concentration of 0.025 U/ml. In contrast, a major portion of the dense granule components were released by thrombin concentrations between 0.02 and 0.05 U/ml with only 15-20% of these components being released at 0.025 U/ml. An increased spontaneous release of alpha-granules would then also give an increased release of PDGF. PDGF stimulates proliferation, uptake of LDL and an increased synthesis of collagen in arterial smooth muscle cells (55,97). With the same method for the spontaneous efflux of platelet substances as used in the present study platelet supernatant has also been demonstrated to increase collagen production when added to growth arrested myocytes and fibroblasts (98). All these reactions in arterial smooth muscle cells seem to be key-events in the development of artherosclerosis (99). The importance of an eventually increased spontaneous release of growth stimulating factors in vivo could not be evaluated with the methods presently available, but it might be of key importance for the development of atherosclerosis in hypertension, at least at the sites of increased endothelial permeability.

Effects of aggregating and desaggregating substances on the spontaneous efflux of norepinephrine and bTG (V)

Thrombin, in increasing concentrations, from very low (0.01 U/ml) to high enough to induce a marked release reaction (0.625 U/ml), added to platelets resuspended in buffer, did not influence the basal release rates of NE. It did, however, give an immediate release of NE and bTG, in amounts which increased at higher doses of thrombin. Thereafter the release curve was parallel with the control curve. Acetyl salicylic acid, which inhibits cyclooxygenase activity and thereby TxA_2 synthesis as well, did not affect the NE efflux curve, either ex vivo or in vitro experiments (Fig 4). Prostaglandin E_1 (PG E_1) (fig 5) and prostacyclin, in concentrations that gave a thirty-fold increase of cAMP accumulation, decreased significantly the initial efflux rate of NE (0-20 min) but the slope calculated on the 30-80 min interval was entirely parallel to that of the control curve. The decrease was most pronounced in those with a basal high efflux rate.

PG E_1 and prostacyclin inhibit the effects of aggregating agents on platelets. This inhibition is mediated by cAMP (100,101). Several reports indicate a direct role for Ca^{2+} in this reaction, probably through its effects on a variety of enzymes today known to be activated by calmodulin (102-104). These enzymes include phosphorylase-kinase, phospholipase A2 and myosin light chain kinase. Haslam et al (105) have shown that PG E_1 enhanced the phosphorylation of platelet membrane polypeptides capable of the ATP-dependent uptake of Ca^{2+}, and they proposed that PG E_1 in this way mediates the active transport of Ca^{2+} out of the platelet cytosol. In spite of the pronounced effect on cAMP with the dose of PG E_1 used in our experiment, the influence on the release of NE was rather slight and transitory. However, there was an obvious effect that was not seen when TxA_2 synthesis was blocked. This might indicate that spontaneous alpha-granule release is sensitive to changes in intracellular Ca^{2+} level, but independent of TxA_2.

The relationship between spontaneous efflux of norepinephrine from platelets, synaptosomes and heart tissue on rats (VI)

To further evaluate the platelet as a model for the neuronal release of NE, the spontaneous release of ^3H-NE was studied in platelets, synaptosomes and heart tissue from the same rat. The different preparations were incubated with ^3H-NE and the release was measured for 20 min. The shorter time used in this study than in all the other studies was due to the very rapid efflux curves from the synaptosomes, where a flattening of the curve was seen already at 20-30 min. During this short time interval the efflux curves were well

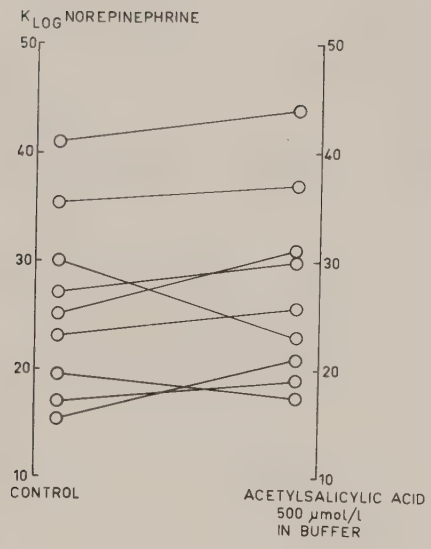

Fig 4.

k value in aliquots where acetylsalicylic acid (500 umol/l) was added to one of the samples at start of the efflux study. (n=9).

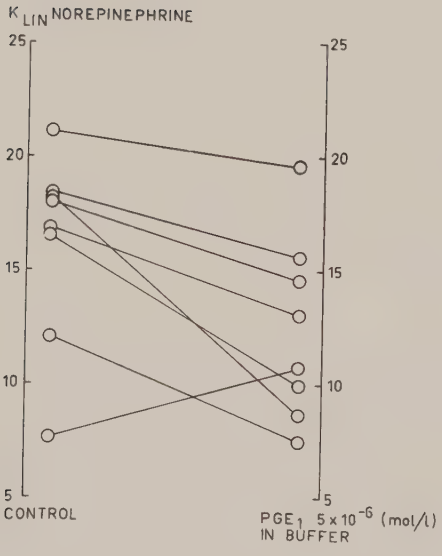

Fig 5.

Linear slope (0-20 min) of NE efflux in aliquots where prostaglandin E_1 ($5x10^{-6}$ mol/l) was added to one of the samples at start of the efflux study. (n=8).

fitted to a straight line in all three systems. The comparatively slow efflux in platelets gave a rather low number of measured CPM during the 20 min study in many cases, making a logarithmic calculation less suitable in this study. The possibility to make 60-80 min efflux studies in platelets as in the earlier investigations was restricted by the low blood volume available in one rat. The slope of the efflux curve corrected for uptake value or, in the heart preparation for tissue weight, (k_{lin}) was used as an expression of the efflux rate.

There was a high correlation between k_{lin} from platelets and synaptosomes ($r = 0.673$ $p<0.001$ $n = 30$) and between platelets and heart tissue in a subsample ($r = 0.549$ $p<0.05$ $n = 16$). These results seem to further strengthen the evidence for a parallel behaviour in platelets and neuronal tissue with respect to the release of NE. This was indirectly demonstrated in the study on DOCA- and salt-induced hypertension in rats (paper III).

GENERAL DISCUSSION

Data from this study seem to point to an increased exocytosis of platelet alpha granules in patients with hypertension and in about 30% of young normotensive relatives of hypertensives.
There also seems to be some parallelism indicated between the release of NE in platelets and in neuronal cells in rats, where it has been demonstrated that a good correlation exists between the efflux in platelets, synaptosomes and heart tissue. As well there is a rise in NE efflux in DOCA and salt-treated rats where earlier investigations have demonstrated a similar condition in sympathetic innervated tissue. Neuronal cells have efficient systems for inactivation of norepinephrine by reuptake and intraneuronal degradation by MAO. Prejunctional alpha$_2$-receptors also exist, the activation of which suppresses further NE release, serving as a negative feed back mechanism. It is impossible to test the influence of these factors in the platelet model and it is unknown whether or not these regulating factors would compensate for an increased basal efflux of NE in neuronal cells. However, if the increased basal efflux is genetically determined and exists already at a very early age, these mechanisms might not be involved, since the unstimulated efflux then would be a basal characteristic for that specific individual. The same could be speculated about the influence of regulation of catecholamine synthesis rate, which could not either be tested in the platelet model.

Data from the study on relatives of hypertensive patients indicate an abnormality in granular NE efflux, which exists before the blood pressure rises. Is it then possible that this mechanism could result in hypertension several years later? If this abnormality would lead to an amplifiquation in the release of transmittor from the terminals at varying rates of neuronal impulse flow, it is likely that it would lead to a sustained arteriolar constriction in several vascular beds. The adaptive changes which result in vascular hypertrophy and finally a persistent increased blood pressure have been investigated by Folkow et al and were proposed to be an important pathogenetic mechanism in essential hypertension (106). Sympathetic nerves increase the rate of synthesis of contractile proteins in vascular muscle (107). In rats, at least, this leads to enhanced vasoconstriction. Sympathetic denervation reduced the rate of thymidine incorporation in vascular muscle, the rate of growth and hypertrophy with age and the wall to lumen ratio of vessels (107,108). Unilateral superior cervical ganglion-ectomy in

spSHR reduced the wall-to-lumen ratio of vessels in the ipsilateral side of the brain as compared to those of the contralateral side, despite the fact that both sides of the brain were exposed to the same arterial pressure (109). By cross transplantation of vessels between SHR and WKR to the anterior eye chamber Hermsmeyer et al (110,111) have demonstrated that the abnormality in membrane permeability of arterial muscle in SHR may be triggered by the nervous system. Thus it seems likely that a trophic sympathetic influence exists in the genetically hypertensive rat which triggers a membrane abnormality leading to hyperactivity, increased permeability, a reduction in membrane potential, exaggerated vasoconstriction and finally hypertension. PDGF has a growth stimulating effect on myocytes and fibroblasts in culture. The present study seems to point to an increased release of PDGF in hypertension. If this phenomenon exists in vivo and has any effects on the vessel wall with intact endothelial lining, remains to be demonstrated.

What could be the mechanism behind the increased exocytosis rate that we have registered? Increased sodium concentration in the incubation medium and DOCA-salt treatment in rats increased the efflux rate. On the other hand, the increase of intracellular sodium registered during the handling of the platelets did not interfere with the k-value. In this latter case the values of platelet Ca^{2+} were very stable, which may be of great importance. It is possible that the variations in the free intracellular Ca^{2+}-level are the important regulating factor, and that Na^{+} exerts its effect through its influence on the Ca^{2+}-level, as an increased concentration of Na^{+} intracellularly induces an increase of intracellular Ca^{2+}-levels. The decrease of platelet norepinephrine efflux at cAMP stimulation also favours the theory of Ca^{2+} as an important regulator. Studies on the relation between intracellular Ca^{2+} concentration and degree of exocytosis of dense bodies does not give evidence for a threshold value of Ca^{2+} giving a total release reaction but furthermore for a gradual increase of exocytosis at increasing intracellular Ca^{2+} concentrations (103).
Postnov et al have demonstrated, in rats as well as in man, that enlarged Ca-pools occur in adipose tissue in hypertensive subjects. In rats it was also shown that there was decreased Ca^{2+}- binding to plasma membranes, a decreased Ca^{2+}-accumulating ability of the endoplasmatic reticulum but an enhanced Ca^{2+} accumulation by mitochondria. Their interpretation was that, since the Ca^{2+} pool in mitochondria is much larger than all other pools, an increased content of intracellular Ca^{2+} was due mainly to increased storage in the mitochondria, caused by a permanent excess of free Ca^{2+}, which

in turn would be due to insufficient Ca^{2+} accumulation by the endoplasmatic reticulum and plasma membrane. An increase in Ca^{2+} concentration then supresses the activity of membrane adenylate cyclase, which was inhibited in adipocytes of hypertensive rats (112,113).

Increased values of intracellular Na in hypertension and in relatives to hypertensives have been reported by several investigators in red and white blood cells (114-119). However, contradictory reports also exist (120-122). The Na/K co-transport was reported to be low in hypertensives and part of relatives to hypertensives (123) and the Na/Li countertransport to be high in the same categories (120). These experiments have been reundertaken by several groups and very contradictory results are reported. There are confirmatory studies, however with a much larger overlap between the groups than was initially reported (124-126). Some studies fail to find any differences between the groups (127-129). These discrepances might in part be ascribed to variations in methodology, and perhaps more important, in the choice of study groups. However, most studies still report differences in sodium transport between hypertensives and normotensives and in part of relatives to hypertensives. (121,130,131). Evidence of membrane abnormality in many different kinds of cells (erythrocytes, platelets, smooth muscle cells and synaptosomes) in SHR have been presented (132,133). The decreased binding of Ca^{2+} inside the plasma membrane also indicates abnormalities in membrane composition. This defect was reported to correlate with the passive permeability for Na^{+} and K^{+} (112).

The role of the Na/K-ATPase activity is also strongly debated. No differences between normo-and hypertensive individuals were initially reported (134), but a recent paper has, with a modification of earlier technique of membrane preparation, demonstrated a decreased Na/K-ATPase activity in erythrocytes of hypertensives (135). Potassium depletion in rats led to a pronounced and reversible decrease in the total number of ouabain binding sites in muscular tissue (136). High sodium/low potassium diets have been discussed as an etiological or additive factor in the development of hypertension for decades. Epidemiological, as well as experimental, data seem to support such theories (137-140).

Taken together all these data point to a membrane defect in hypertension which leads to abnormalities in the cellular handling of Na^{+} and Ca^{2+}, which might in turn induce increased vascular reactivity and disturbances in the regulation of nerve transmission.

Protein carboxyl-methylation have been proposed to play an important role in the regulation of exocytosis and chemotaxis (141). This system is composed of three main elements: a methylating enzyme, protein carboxylmethylase (PCM), a demethylating enzyme, protein methylesterase (PME) and the substrate, methyl acceptor proteins (MAP). PCM specifically methylates certain proteins containing aspartic or glutamic acid residues and it is the free carboxyl groups of these residues which are methylated. The best substrates for PCM in the organelle containing organs are the secretory granule membranes. Most of the PCM activity is distributed in the cytoplasm. It is supposed that MAP in the granular membrane are methylated by PCM and that the negatively-charged granule membrane system is thereby neutralized. The probability of collision between the granules and the negatively charged inner surface of the cell plasma membrane would then favour the start of the exocytosis process (142). Furthermore, calmoduline has been reported to be an exceptionally good substrate for PCM. Its methylation inhibits the stimulatory effects on the Ca^{2+}-dependent enzyme cyclic nucleotide phophodiesterase. It is possible that other Ca^{2+}-dependent processes, such as granular exocytosis, could also be regulated by calmodulin methylation and it might be a possibility that the degree of methylation of calmodulin might sensitize granular exocytosis at lower levels of cytosolic Ca^{2+} than would otherwise be needed.
Studies on the activity of PCM and PME in platelets from relatives to hypertensives are in progress. Only data concerning PCM activity are hereto available. No significant difference in PCM activity was seen between normotensive relatives to hypertensives and controls. Studies on PME-activities and MAP are still in progress.

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THE EFFLUX RATE OF NOREPINEPHRINE FROM PLATELETS AND ITS RELATION TO BLOOD PRESSURE★

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Summary

The uptake and efflux rate of norepinephrine in platelets have been studied in 63 individuals, 48 men and 15 women. 40 of these had a diastolic blood pressure ≥95 mm Hg. If the initial efflux rate, k, was correlated to the diastolic blood pressure a highly significant relationship ($r = 0.748$ $p<0.001$) was obtained, i.e. a high diastolic blood pressure is correlated to a rapid efflux of norepinephrine from platelets. No correlation was found between uptake values and diastolic blood pressure.

The role of the sympathetic nervous system in the development and maintenance of high blood pressure is, in spite of many years of research, still unclear. After development of sensitive methods for measurement of norepinephrine in plasma several investigations about the interrelation of plasma levels of norepinephrine and blood pressure have been published (1-7). The basic hypothesis is, that increased sympathetic activity leads to an increase in the release of catecholamines from nerve terminals which should be reflected in a rise in the plasma norepinephrine concentrations. The results from these investigations are however conflicting. The complicated processes at the nerve terminal - secretion, reuptake, metabolism - make interpretation of plasma norepinephrine level as an expression of symphathetic nerve activity hazardous.

Direct studies of the norepinephrine turn over in sympathetic nervous tissue in rats have shown that hypertension induced by the administration of desoxycorticosterone and sodium chloride is accompanied by a decreased capacity to retain norepinephrine in the storage granules, leading to a constantly increased outflow of norepinephrine from the neuron (8). This deficient storage capacity was seen before the blood pressure started to rise and normalization occurred when the salt loading was interrupted with subsequent normalization of blood pressure.

According to this research model an increased efflux of norepinephrine from the storage granules seems to be fundamental for the development of high blood pressure. It should be of great value to find a model system enabling

★A preliminary report of these data was presented at the 35th annual meeting of the Swedish Medical Association at Stockholm (36).

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the study of the norepinephrine efflux from neurons in essential hypertension in man. Platelets have been extensively studied as a model for adrenergic neurons and have been shown to have essential properties in common with the neuron (9-11). They can accumulate norepinephrine against a concentration gradient (12), the amine is stored in analogous subcellular organelles in the platelets and in the nerve terminals (13,14) and the transport system in platelets and in nerve cells show a number of identical characteristics (12, 15-22).

These observations have been utilized in the studies on some neurological and psychiatric diseases with known alterations of the biogenic amines in the central nervous system, i.e. endogenous depression (23,24) and Parkinson's disease (25-27). No previous studies have been published on the accumulation and release of norepinephrine in platelets in essential hypertension. The intention of the present study is to find a possible correlation between the efflux rate for norepinephrine from platelets and the blood pressure level in human subjects.

Patients

Blood samples were taken from 63 individuals. Fourtyeight of these were 48-49 year old apparently healthy males undergoing a health control examination at the Department of Preventive Medicine. This examination was offered to all men in Malmö of the same age cohort. Fifteen were women, 8 of them with diastolic blood pressure 105-150 mm Hg. The mean age of these 8 subjects was 50.3 (29-69). Seven women had a diastolic blood pressure ≤90, the mean age of these was 45.6 (33-58). All the individuals were free from all medication. The blood pressure was measured by trained nurses in the morning, recumbent after exactly ten minutes rest, and the blood sample was taken on the same day. A mercury manometer was used and as diastolic blood pressure the disappearance of the Korotkoff sounds was used (phase V).

All individuals with a diastolic blood pressure ≥105 (n = 24) have later been examined thoroughly at the Hypertension Unit of the Medical Department. They all received a careful physical examination and sodium, potassium, calcium, creatinine, uric acid, liver enzymes, haematological parameters and lipids in blood were analyzed. Only in a few selected cases with extreme hypertension were intravenous pyelography or renal angiography performed and then showed no signs or suggestions of primary renal disease. These 24 individuals were labelled as having essential hypertension.

Materials and methods

14C-norepinephrine [DL(carbinol - C14) noradrenaline DL bitartrate, 37 mCi/mmol] was purchased from Amersham and 3H-serotonin [hydroxytryptamine binoxalate 5-{1,2 - 3H(N)} 25.1 Ci/mmol] from New England Nuclear.

Venous blood was collected after >10 hours fast in the morning in 10 ml siliconized tubes, each containing 1 ml NaCl 0.12 M and EDTA 0.027 M and were immediately cooled on ice. Within an hour the tubes were centrifuged (120 x G for 15 minutes at 4° C), platelet rich plasma (PRP) was separated and cooled on ice until the incubation started. Platelet counts were done in duplicate using a celloscope (Technicon) and the mean value obtained was used for calculation.

Two separate portions of PRP were incubated in an atmosphere of 95% O_2/5% CO_2 at 37°C in a Dubnoff metabolic shaker. 14C-norepinephrine and 3H-serotonin were added to give 0.1 μCi/ml PRP (2.7×10^{-6} M) and 0.02 μCi/ml PRP (7.96×10^{-8} M), respectively. The incubation was continued for 90 minutes and then stopped by cooling on ice. Platelets were isolated by centrifugation (8 000 x G for 5 minutes at 4°C) and resuspended in Krebs-Ringer bicarbonate buffer (pH 7.4) from which calcium and magnesium had been omitted. Two aliquots were taken from the samples immediately after the resuspension, one for determination of the uptake and one to give a zero-time value. Thereafter the platelet suspension was again incubated as above. Aliquots (0.5 ml) were taken at regular time intervals, every 10th minute during the first hour and every 20th minute during the second hour. After 120 minutes the incubation was stopped. The aliquots were centrifuged immediately after being taken from the incubation (10 000 x G for 10 minutes at 4°C) and a fixed volume of the supernatant was decanted into liquid scintillation vials containing 10 ml Instagel (Packard Instr. Comp.). Radioactivity was determined by liquid scintillation spectrometry using a Packard liquid scintillation spectrometer B 2450. Quench correction was made by external standard. For the determination of the uptake of norepinephrine and serotonin the platelets were disrupted by sonification (Branson Sonic Power Company B-12, 30 seconds at setting 6) before scintillation counting.

To test the potential influence of variations of the endogenous plasma norepinephrine concentrations L-noradrenaline-L-tartrate (Merck) was added to PRP to a final concentration of 0.1, 1 and 10 ng/ml before the incubation. No change in the efflux rate of 14C-norepinephrine was registered, and this step was later omitted.

To test the influence of different sodium ion concentrations on uptake and efflux of norepinephrine and serotonin, buffers were used with various sodium chloride concentrations in the interval 110-170 mM but in all other aspects of identical composition with the stock Krebs-Ringer bicarbonate buffer. PRP was centrifuged (8 000 x G for 5 minutes at 4°C) to separate the platelets. These were resuspended in buffers with different sodium ion concentrations, incubated in carbogen atmosphere at 37° in a metabolic shaker for 30 minutes. Thereafter 14C-norepinephrine and 3H-serotonin were added in the same concentrations as above and incubated for 90 minutes. The platelets were then again separated by centrifugation (8 000 x G for 5 minutes at 4°C) and were resuspended in a buffer with the same sodium ion concentration as used in the incubation. Aliquots for determination of uptake and efflux of norepinephrine and serotonin were taken at regular time intervals.

Results

Counts obtained in the supernatant were corrected for background activity by subtracting the zero-time value. In a semilogaritmical plot of these values for efflux against time a linear relationship was obtained for the whole time of observation when the efflux was slow. When the efflux was rapid the curve was initially linear and thereafter a marked decrease in efflux rate was seen (fig. 1). The efflux rate used in the following discussion, k, was defined by the slope of the first linear part of the curve, and was calculated by regression analysis using the formula:

$$k = \frac{\Sigma x \ln y - \frac{1}{N} (\Sigma x)(\Sigma \ln y)}{\Sigma x^2 - \frac{1}{N} (\Sigma x)^2} \times 10$$

where y is CPM measured and x is time in minutes. This slope was taken as a measure of the initial efflux rate. For each curve the correlation coefficient for the values on the linear portion was also calculated. If this was less than the arbitrary value 0.85, the curve was omitted from the material since obviously some experimental errors could be contributing. The mean correlation coefficient for the curves in the total material was 0.95 ± 0.04 S.D.

If the initial efflux rate k for norepinephrine from platelets is correlated to the diastolic blood pressure a highly significant relationship is obtained (coefficient of correlation = 0.748 $p < 0.001$) (fig. 2). This indicates that high diastolic blood pressure is correlated to a rapid efflux of norepinephrine from platelets.

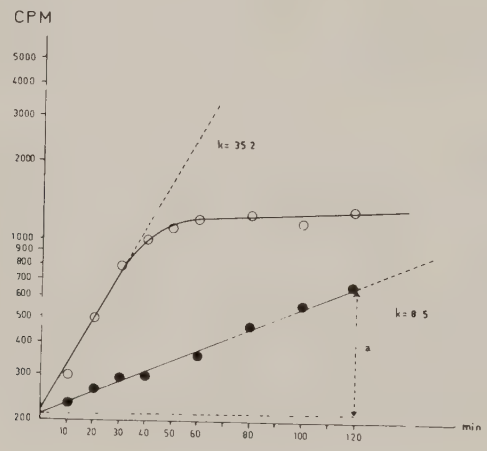

Fig. 1

The efflux of 14C-norepinephrine from platelets as a function of time on two different individuals. The rate coefficient for the initial phase of efflux is calculated from the formula given in the text. A good estimation of the k-value is obtained by measuring the distance "a" in the figure.

An easier, though less objective way of calculating the slope of the efflux curve is to take the ordinate of the extended linear portion of the curve at 120 minutes corrected for the zero-value (fig. 1).

Of 15 women studied 7 had a diastolic blood pressure ≤90. Tests on these gave a mean k-value = 13.9 ± 2.6. The mean k-value on 16 men with diastolic blood pressure ≤90 was 10.4 ± 4.5 ($p = 0.05 - 0.025$).

In five cases determinations of k-values were made on two different occasions with 1-2 weeks interval. The reproducibility for each individual was good, the mean difference in k-value being 2.98.

To test if a large variation of endogenous blood levels of norepinephrine could influence the efflux rate of norepinephrine from platelets non-radioactive norepinephrine was added at the incubation step to the ordinary concentrations of labelled norepinephrine in concentrations 0.1 - 10 ng/ml which correspond to the reported endogenous levels even at extreme hypertension (2-4). This did not influence the efflux rate of norepinephrine.

The uptake of norepinephrine was measured in 27 of the individuals with diastolic blood pressure between 80 and 150 mm Hg. No correlation was found between the diastolic blood pressure and the uptake values for norepinephrine, nor between the uptake and the k-value. The mean uptake was 877 ± 372 DPM/10^{11} platelets.

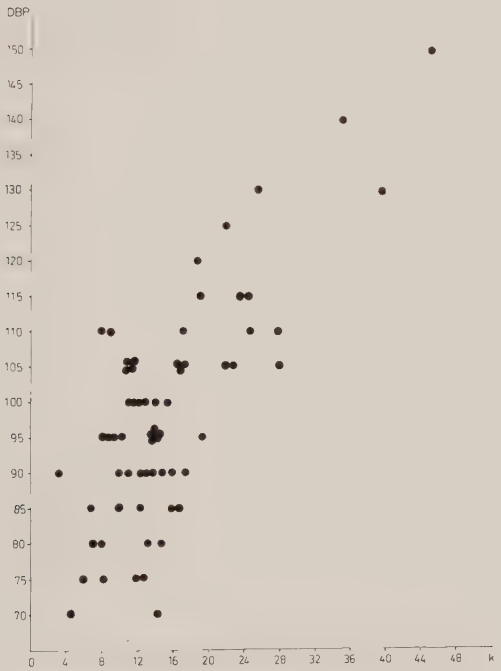

Fig. 2

Graphical illustration of the relation between diastolic blood pressure and efflux rate coefficient for 14C-norepinephrine from platelets taken from 63 individuals. ($r = 0.748$, $p<0.001$)

3H-serotonin was added to serve as a marker to detect eventual aggregation, as it has been established that platelets release all accumulated serotonin within a few minutes of aggregation (28). The 3H-serotonin added gave uptake values that were about 25% of those for 14C-norepinephrine measured in DPM. The efflux of 3H-serotonin was very slow and not parallell to the efflux of norepinephrine. The serotonin added did not influence the efflux rate of norepinephrine.

Increasing concentrations of sodium ions in the platelet incubation buffer gave increased rates of efflux for each concentration level in the interval of 110-170 mM. Results obtained from one such experiment are shown in fig. 3. A more detailed description of the influence of sodium as well as of other factors influencing the efflux rate of norepinephrine from platelets is under preparation.

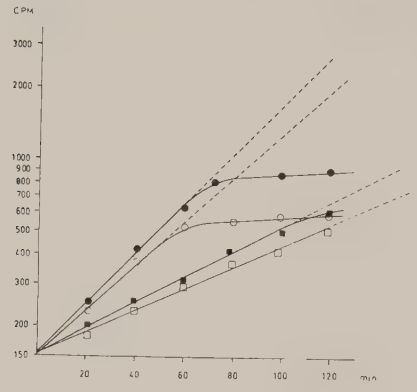

Fig. 3

The efflux of 14C-norepinephrine from platelets as a function of sodium ion concentration in the external medium. A platelet rich plasma was divided into identical portions that were exposed to varying concentrations of sodium chloride.

□= 110 mM Na^+ ■= 130 mM Na^+ ○= 148 mM Na^+ ●= 170 mM Na^+

Discussion

In the present study it has been shown that in human essential hypertension there is a significant correlation between the diastolic blood pressure and the initial rate of efflux of norepinephrine from the platelets. The observed pattern, with a linear relation between efflux and time in a lin-log plot indicates that the mechanisms behind the observed effects may be complex and may be a function of several interacting steps. It has been suggested (29,30)that there may exist two different pools of norepinephrine in the same cell. Furthermore, kinetic behaviour with pronounced lag phases is often observed when two or more steps interact (31).

In the study on retention of 3H-norepinephrine in heart tissue from rats treated with DOCA and sodium chloride there was found a significant inverse correlation between the systolic blood pressure and the accumulation of 3H-norepinephrine in heart (8). This means that the efflux of norepinephrine from heart tissue must have been faster at high blood pressure. There is a striking similarity between these results obtained in experimentally DOCA and salt induced animal hypertension where sympathetically innervated tissue was studied and those found in our studies on humans where platelets were used as a model system. Differences in uptake of norepinephrine were not observed in either the animal study nor in the present study.

The modified storage capacity for norepinephrine in heart tissue from rats was shown to be situated in the microsomal fraction, i.e. in the storage granules for norepinephrine of the neuron. In platelets accumulated catecholamines are enriched in the microsomal fraction. In this fraction there has been found a mucopolysaccharide-protein complex capable of binding biogenic amines as well as sodium ions (32). A similar mucopolysaccharide-protein complex was also found in adrenal medullary cells (33) and in adrenergic vesicular fractions from sympathetically innervated tissues, peripheral sympathetic nerves and brain (34). There may exist a situation of interference between biogenic amines and cations. In the study on induced hypertension in animals salt loading causes a decreased storage capacity for norepinephrine. This appeared before the blood pressure started to rise and when DOCA-salt feeding was interrupted the storage capacity returned to normal values. Thus it seems reasonable to assume that the sodium ion plays an important role in the creation of an increased efflux of norepinephrine from storage granules of the neurons.

Preliminary experiments on platelets show that increasing the concentration of Na^+ in the medium result in faster efflux of norepinephrine (manuscript in preparation).

A decreased total turnover of $^{22}Na^+$ has been observed in normotensive subjects belonging to families with high frequencies of hypertension (35). Studies on the efflux rate of norepinephrine from platelets compaired with the total body turn over of $^{22}Na^+$ has just been started in this same population. This also seems to stress the importance of sodium ions for the development of hypertension in man.

Acknowledgements

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Efflux of noradrenaline from platelets in normotensive members of hypertensive families

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Summary

1. Platelets have been used as a model of sympathetic neurons to study the storage of noradrenaline in normotensive individuals belonging to families with essential hypertension for at least two generations.

2. The initial efflux rate (k) of noradrenaline was determined in 44 young relatives (mean age 29·2 years), in 18 middle-aged relatives (mean age 46·7 years) and in 31 young controls with no known family history of essential hypertension (mean age 29·8 years). From the groups of relatives all those with definite hypertension had been excluded *a priori*.

3. k was significantly higher in the young relatives (22·7 ± 7·9) than in the middle-aged relatives (17·7 ± 6·4) and in the controls (15·6 ± 5·1). Of the relatives 27·3% had higher k values than any of the controls. A significant correlation was found between k and diastolic blood pressure in controls but not in young relatives.

Key words: genetic marker, hypertension, inheritance, neuronal mechanisms, platelets.

Introduction

Platelets accumulate and store noradrenaline in subcellular granules in a way that in essential aspects seems to be closely similar to the neuronal storage of noradrenaline [1, 2]. In an earlier study we have shown that in middle-aged individuals there was a close correlation between the efflux rate of noradrenaline from platelets and the diastolic blood pressure. Exposure *in vitro* to increasing Na^+ concentrations gave successively faster net efflux of noradrenaline from platelets [3]. Considerable evidence has accumulated that kinetics of sodium turnover in erythrocytes [4–10] and lymphocytes [11] is different in normotensive and in essential hypertensive subjects. This was also apparent when a large group of normotensive individuals from families with a high incidence of hypertension was compared with controls. The same observations have been made in animal genetic hypertension [12–15]. Animal experiments have shown that sodium loading interfered with the normal storage of noradrenaline in microsomal granules from heart tissue and that this deficient storage developed before blood pressure started to rise, with subsequent return to normal when salt loading was interrupted and blood pressure returned to normal levels [16].

This study concerns the net efflux rate of noradrenaline from platelets in normotensive individuals from families with a high incidence of essential hypertension.

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Study population

Group 1: controls were 31 young individuals (21 males, 10 females) between 22 and 40 years (mean 29·8 years) who on interview in depth were definite in that there was no hypertension among their relatives for two generations. Blood pressure was ≤135/85 (mean 120·5/73·7) mmHg.

Group 2: 44 young individuals (31 males, 13 females) between 18 and 40 years (mean 29·2 years). They all had a first-degree relative with essential hypertension, which was verified by interview and examination by a physician. By interview it was established that hypertension

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developing before the age of 60 existed in the family for at least two generations, and was not associated with evidence of renal disease or diabetes mellitus. The incidence of hypertension in these families was close to 50% [17]. The individuals belonged to 39 different families.

Blood pressure was ≤145/100 (mean 124·2/81·9) mmHg at the day of blood sample collection. Four individuals had diastolic blood pressure ≥95 mmHg; their blood pressure was taken again 2 days later and was then ≤90 mmHg. However, for all calculations the first measured pressure was used.

Group 3: 18 middle-aged relatives (10 males, eight females) between 41 and 52 years (mean 46·7 years) with the same criteria of inheritance as above, belonging to 16 families. Blood pressure was ≤ 155/100 (mean 121·3/81·6) mmHg. One individual had a diastolic pressure of 100 mmHg; the rest had diastolic pressures ≤ 90 mmHg.

Blood samples were taken for tests on liver enzymes, creatinine, electrolytes, serum albumin and for determination of efflux rate of noradrenaline from platelets. All individuals were on a free diet, were receiving no medication (including contraceptive drugs) and had serum γ-glutamyltranspeptidase within the normal range (≤1·0 μkat/l).

Methods

Blood pressure was measured in the horizontal position by the same nurse in the morning after rest for exactly 10 min. A mercury manometer was used, and the disappearance of the Korotkoff sounds was taken as diastolic blood pressure (phase V).

Determination of the net efflux rate of noradrenaline from platelets was made according to a method described earlier [3]. Platelet-rich plasma was incubated with [^{14}C]noradrenaline (0·1 μCi/ml of platelet-rich plasma = $2·7 \times 10^{-6}$ mol/l) for 90 min; when uptake had reached equilibrium platelets were isolated by centrifugation (3000 *g*, 10 min) and resuspended in Krebs–Ringer bicarbonate buffer from which Ca^{2+} and Mg^{2+} had been omitted. Efflux was determined in aliquots taken every tenth minute for 60 min and in one after 80 min. Radioactivity was determined by liquid scintillation counting. In a semilogarithmic plot of ^{14}C efflux (c.p.m.) against time a linear relationship was obtained for the first phase of the efflux process. The efflux rate, *k*, was defined by the slope of the linear phase and was calculated by linear regression. *k* was calculated on values

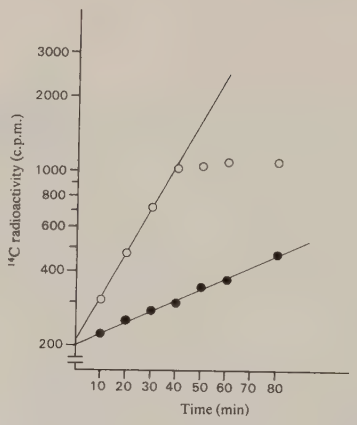

FIG. 1. Net efflux of [^{14}C]noradrenaline from platelets (of two different individuals) as a function of time. Regression coefficient (*k*) is calculated on the initial linear part of the curve.

obtained during 60 min with the exception that, in very rapid efflux, where a marked decrease of efflux rate was seen before 60 min, *k* was calculated on the linear part of the curve; after the last calculated value at least two values that had changed less than 10% (Fig. 1) were required, to indicate that the initial rapid efflux had started to reach a plateau. Mean coefficient of correlation (*r*) for all *k* value calculations was 0·95 ± 0·04.

The significance test between the three experimenal groups was performed by means of analysis of variance. For comparison between two groups Student's *t*-test was used. All mean values are given with ± 1 SD.

Results

Mean systolic blood pressure did not differ between controls and young relatives (Table 1), but mean diastolic blood pressure was significantly higher in the relatives ($P < 0·001$ for young relatives, $P < 0·01$ for middle-aged relatives). The efflux rate of noradrenaline, *k*, was significantly higher in the young relatives than in the controls (22·7 ± 7·9 vs 15·6 ± 5·1, $P < 0·001$). In the older relatives *k* was significantly lower (17·7 ± 6·4, $P < 0·05$) than in the young relatives (Table 1). Within the group of young relatives was a subgroup of individuals with very high *k* values in spite of normal blood pressure (Fig. 2); 27·3% of these had *k* values higher than

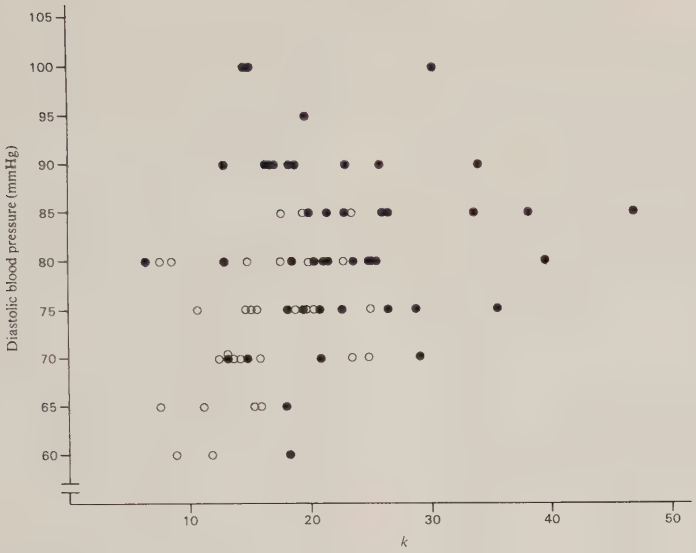

FIG 2. Relation between diastolic blood pressure and k in controls (○) and young relatives (group 2) (●).

TABLE 1. *Age, blood pressure and k values in relatives and controls*

Mean values ± 1 SD are shown. Ranges of ages are given in parentheses.

	Age (years)	Blood pressure (mmHg)	k
Controls (M = 21, F = 10)	29·8 ± 5·2 (22–40)	120·5 ± 8·8/ 73·7 ± 7·1	15·6 ± 5·1
Relatives ⩽40 years (M = 31, F = 13)	29·2 ± 6·1 (18–40)	124·2 ± 11·2/ 81·9 ± 9.0	22·7 ± 7·9
⩾40 years (M = 10, F = 8)	46·7 ± 3·4 (41–52)	121·3 ± 13·8/ 81·6 + 8·1	17·7 ± 6·4

any of the controls (Fig. 3). No correlation was seen between diastolic blood pressure and k in young relatives. In the controls, however, there was a significant correlation between diastolic blood pressure and k ($r = 0{\cdot}39$, $P < 0{\cdot}05$). No difference in k was seen between males and females in any of the groups. No significant difference between values for uptake of [^{14}C]-noradrenaline was seen in any of the groups.

The group of young relatives was divided into two subgroups according to diastolic blood pressure: one with diastolic blood pressure ≤ 80 mmHg ($n = 24$) and one with diastolic blood pressure ≥ 85 mmHg ($n = 20$). Mean blood pressure was 122·7/75·4 mmHg and 126·0/89·8 mmHg respectively. No difference in mean k value was seen between the two subgroups (21·7 ± 7·0 vs 23·8 ± 8·9) and mean k in both groups was significantly higher than in the control group ($P < 0{\cdot}001$).

If the young relatives were matched with a control subject according to sex, age and diastolic blood pressure with an accepted variance of ± 5 mmHg, 27 pairs could be formed. The mean k value for the relatives was significantly higher than for the controls ($P < 0{\cdot}001$) (Table 2).

Discussion

In the present study it is shown that in a group of young relatives belonging to families with a high incidence of essential hypertension 27·3% have efflux rates of noradrenaline from platelets that are higher than for any of the controls, and that the whole group has a deviation towards higher rates of efflux. A close correlation between k and diastolic blood pressure was shown in an earlier study [3]. These data suggest that the relatives with the high k values are those who are going to be hypertensive and that k is a marker for genetic hypertension that could be measured before blood pressure starts to rise. If this hypothesis is correct, high k values should be infrequent in normotensive, middle-aged relatives. This was the reason for studying also a group (group 3) of

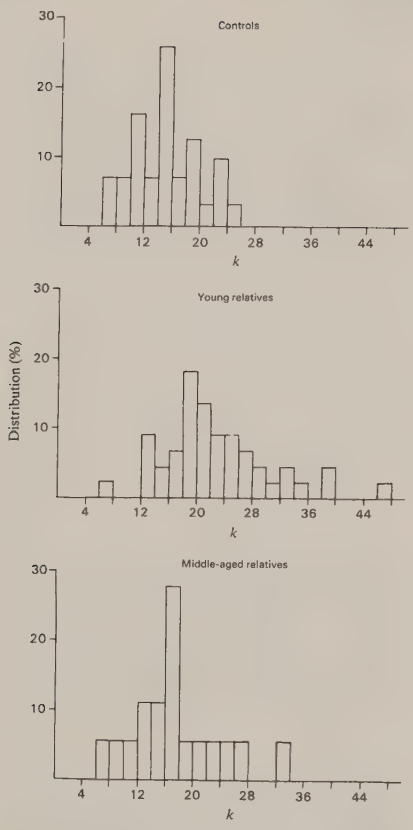

FIG. 3. Distribution of k in 31 young control subjects (≤ 40 years) with no inheritance of hypertension, in 44 young (≤ 40 years) and in 17 middle-aged (40–52 years) individuals who are first-degree relatives to an individual with essential hypertension and have had hypertension in the family for at least two generations.

TABLE 2. *Comparison of k values in two groups of young relatives and controls, matched according to sex, age and diastolic blood pressure*

Mean values ± 1 SD are shown. N.S., Not significant.

	Controls	Relatives
Age (years)	29·6 ± 5·4	27·7 ± 5·8 N.S.
Blood pressure (mmHg)		
Systolic	120·7 ± 8·4	124·6 ± 10·9
Diastolic	74·8 ± 6·6	77·0 ± 6·5 N.S.
k	16·2 ± 4·5	23·3 ± 8·0 ($P < 0{\cdot}001$)

subjects in this category. We found that k was significantly lower in the 18 individuals between 40 and 52 years than in the young relatives and not different from that of the controls. However, the only reliable way to test the hypothesis that this is a genetic marker is to carry out follow-up studies. These are in progress.

Recent data from several groups conclude that the kinetics of sodium turnover is disturbed in different types of cells from normotensive members of families with essential hypertension. An inherited defect in Na^+/K^+ cotransport in erythrocytes has been demonstrated [6]. The net influx of ^{22}Na in erythrocytes was higher [7] and the Na^+/Li^+ countertransport faster [4]. The ratio of net Na^+ and K^+ fluxes in sodium-loaded erythrocytes was lower because of a high potassium influx]5], and the intracellular erythrocyte concentration of sodium was higher in relatives than in controls [8, 9]. Heavy alcohol consumption has also been shown to increase intracellular concentrations of sodium [18]. All individuals in the present study, however, had normal γ-glutamyltranspeptidase values and on interview denied high consumption of alcohol. High γ-glutamyltranspeptidase activities ($> 1{\cdot}45$ μkat/l) have on interview in depth been associated with heavy consumption of alcohol [19].

In view of our earlier findings that over a broad range of sodium concentrations in the medium the net efflux increased with higher sodium concentration [3], it seems possible that the increased net efflux rate of noradrenaline from platelets that we have found in the present study might be caused by disturbances like the change in sodium metabolism in relatives discussed above. Whether there also exists an increased net efflux rate of noradrenaline in synapses and nerve terminals needs more investigation. Our own results show that treatment of rats with DOCA and extra salt in drinking water causes significantly higher rates of efflux of noradrenaline from platelets [20]. DOCA and salt treatment have earlier been shown to decrease noradrenaline in the granular fraction from heart tissue in rats [16]. These data strengthen the evidence for parallelism between neurons and platelets as long as platelet aggregation is prevented. The release of noradrenaline from sympathetic nerve terminals seems to be based on exocytosis of storage granules [21]. Recent data of our own also show that noradrenaline is released from platelets by exocytosis of alpha-granules (L. Stavenow, I. Mattiasson, B. Teodorsson & B. Hood, unpublished work). Pletcher *et al.* [2] recently concluded that, with regard to exo-

cytotic release of catecholamines, platelets may be used as models for both serotonin- and catecholamine-neurons. On the other hand, platelets do not seem to be completely satisfactory models for drug-induced amine liberation by monoaminergic neurons [22].

Platelets exhibit no endogenous production of noradrenaline. We have in these experiments supplied [^{14}C]noradrenaline. No difference was seen in uptake values between the groups. There is, of course, no evidence that factors leading to an increased rate of exocytosis in the neurons will necessarily be associated either with parallel increases in all steps of biosynthesis of catecholamines or for that matter with an entirely parallel formation of storage granules *de novo*. Whether the content of endogenous noradrenaline per granule would be the same at widely varying rates of granular exocytosis is unknown.

Acknowledgments

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NET EFFLUX RATE OF NOREPINEPHRINE FROM PLATELETS IN DOCA- AND SALT-TREATED RATS

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Summary

39 Wistar Kyoto rats were treated for 4 weeks with weekly injections of DOCA and extra sodium chloride in drinking water. The efflux rate of norepinephrine from platelets (k) was determined in 13 samples, each consisting of pooled blood from 3 animals. The efflux rate of norepinephrine was significantly higher ($k=24.7\pm4.9$) in these animals than in the 29 controls in which 10 k-determinations were made ($k=17.0\pm4.5$) ($p<0.001$). The heart weight was significantly higher in the DOCA-salt treated group and there was a significant correlation between heart weight and k ($r=0.49$ $p<0.05$). As earlier studies have demonstrated a decrease in norepinephrine in the granular fraction from heart tissue in DOCA-salt treated and hypertensive rats these findings suggest close similarities between norepinephrine storage in platelets and in neuronal cells.

In 1967-1969 de Champlain et al reported a series of studies concerning the retention of norepinephrine in the granular fraction from heart tissue in Sprague-Dawley rats treated with deoxycorticosterone (DOCA) and/or variation of salt intake (1-4). They then demonstrated a decreased retention of isotope labelled norepinephrine in several organs one hour after intravenous injection of the substance in hypertensive rats. They found that the concentration of 14C-norepinephrine was significantly lower in the granular fraction of ultracentrifuged heart tissue in the DOCA-salt treated hypertensive animals than in the controls.

Platelets take up norepinephrine against a concentration gradient (5) and store it in subcellular organelles (6, 7). The uptake and storage of biogenic amines in platelets and in nerve cells seem to have close similarities (8). Platelets have accordingly been used to study the accumulation of biogenic amines in some diseases with known disturbances of these functions in the central nervous system. It has thus been demonstrated that in Parkinsons´ disease the uptake of dopamine in platelets was decreased (9). In endogenous depression platelet serotonin as well as brain serotonin was decreased (10).

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In earlier studies in man we have demonstrated a highly significant correlation between the diastolic blood pressure and the efflux rate of norepinephrine from platelets in middle-aged individuals (11). We have also found that within a group of normotensive individuals belonging to families with a heavy accumulation of essential hypertension there is a subgroup of individuals with very high rates of efflux of norepinephrine (12). Platelets exposed in vitro to sodium ion in the range 110-170 mM have a higher rate of norepinephrine efflux at higher sodium concentrations (11).

The aim of the present study was to assess the net-efflux rate of norepinephrine from platelets in rats treated with DOCA and NaCl to find out whether such treatment, earlier shown to interfere with norepinephrine storage in nerve granules, also interferes with norepinephrine storage in platelets. Clarification of this point appears urgent in order to test the validity of the use of platelets in the study of granular norepinephrine storage in hypertension.

Material and methods

Male Wistar Kyoto rats (WKY), weighing 100-125 g were used for the study. Weekly subcutaneous injections of 0.7 ml of a suspension containing 25 mg DOCA, 10.5 mg methylcellulose, 3 mg carboxymethylcellulose, 1 mg polysorbate 80 and 8 mg NaCl/ml and 1% NaCl solution in tap water ad. lib. for drinking were given to 39 animals for 4 weeks. A control group (n=29) was given no injections and tap water for drinking. Both groups were given a regular laboratory diet (Astra-Ewos, Södertälje, Sweden). When given DOCA-injections and salt in drinking water for more than 4 weeks the animals developed signs of heart failure with peripheral oedemas in the legs and signs of discomfort. At this stage the experiment was discontinued. The rats were sacrified by blunt head trauma and decapitated. Blood samples from 3 animals were pooled (in one k determination in the control group from 2 animals) to obtain enough material for the platelet study. Hearts were immediately removed and weighed.

The net efflux rate of norepinephrine from platelets (k) was determined according to the method described earlier (11). Platelet-rich plasma (PRP) was incubated with 14C-norepinephrine (0.1 µCi/ml PRP = 2.7×10^{-6} mol/l) for 90 minutes, platelets were isolated by centrifugation and resuspended in Krebs-Ringer bicarbonate buffer from which Ca^{2+} and Mg^{2+} had been omitted. The efflux was determined in aliquots taken every 10 minute for 60 minutes and in one after 80 minutes. Radioactivity was determined by liquid scintillation counting. In a semilogaritmic plot of efflux CPM against time a linear relationship was obtained for the first phase of the efflux process. k was defined by the slope of the linear phase and was calculated by regression analysis. k was calculated from values obtained during 60 minutes unless the efflux was very rapid and decreased markedly before the end of this time. In these exeptions k was calculated from the initial linear part of the curve. The last value used in the calculation was the one followed by at least two values that had changed by less than 10%.

For the statistical calculations both Student´s t-test and the Mann-Whitney test were used. p-values given were obtained from Student´s t-test. Linear regression was used to test correlations. Mean values are given with ± 1 S.D.

Results

Thirteen determinations of k were made in DOCA-salt treated rats. The mean value was 24.7±4.9. The mean heart weight (n=30) in this group was 1.18±0.14 g. The mean value of 10 determinations of k in controls was 17.0±4.5 ($p<0.001$) and the mean weight of 29 hearts was 0.87±0.11 g ($p<0.001$) (figure 1). There was a significant correlation between mean heart weight in the three animals used for each k-determination and the k-value found ($r=0.49$ $p<0.05$) (figure 2).

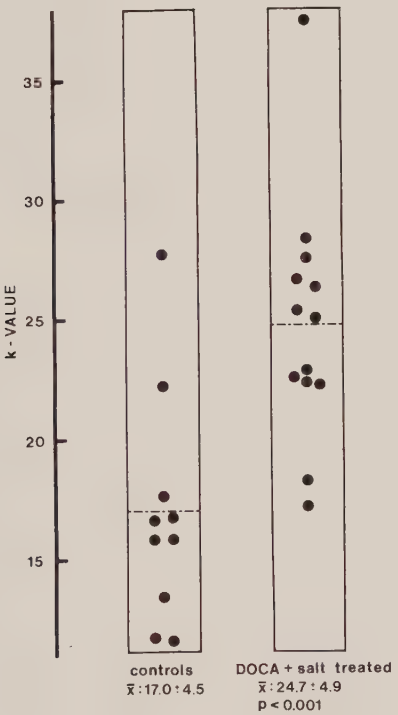

Fig. 1

k-value in Wistar Kyoto rats treated with DOCA and salt for 4 weeks (n=13 from 39 animals) and controls (n=10 from 29 animals). $\bar{X}$=mean ± S.D.

Discussion

The Wistar Kyoto rats (WKY) were chosen for this study because this is now the normotensive strain most widely used in hypertension research. We found however that unlike Sprague-Dawley rats used by de Champlain et al salt loading could not be continued for 6 weeks. After 4 weeks the WKY showed signs of heart insufficiency and oedema. This might indicate that WKY is a more salt sensitive strain than the Sprague Dawley strain. A few personal observations in 9 Sprague Dawley rats showed that these animals thrived and had no signs of oedema after 6 weeks under exactly the same conditions as the WKY strain. The difference found between the strains could thus hardly be ascribed to any difference in the handling of the animals in the two studies.

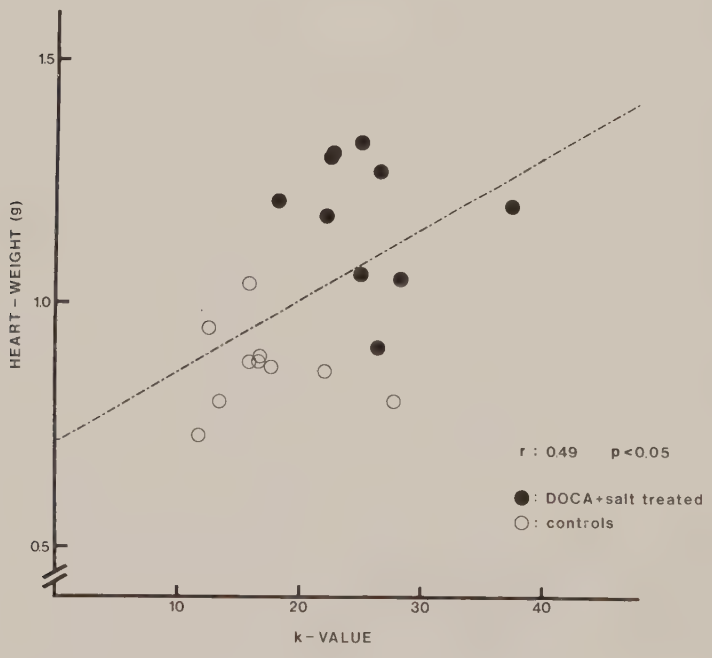

Fig. 2

Relation between mean heart weight in 3 animals and k-value in DOCA and salt treated Wistar Kyoto rats (n=10 from 30 animals) and controls (n=10 from 29 animals). $r = 0.49$ $p<0.05$.

de Champlain et al demonstrated an inverse correlation between ^{3}H-norepinephrine concentration in heart tissue and systolic blood pressure (SBP) as well heart weight (1). The significantly higher heart weight in the DOCA-salt treated group in this study suggests that these animals had developed hypertension. In another study

(13) 4 weeks treatment in WKY with DOCA and salt gave a significant increase in systolic blood pressure. In our study blood pressures were, however, not measured. The direct correlation between heart weight and norepinephrine efflux rate constant reported here may account for the inverse correlation between heart weight and norepinephrine retained in the heart reported by de Champlain et al. They concluded that the treatment with DOCA and NaCl and, though to a lesser degree, with NaCl impaired the capacity to retain norepinephrine in the granular fraction of the sympathetic nerves and as this defect preceded the rise in blood pressure and ceased on discontinuation of the salt-DOCA treatment they felt that this interference by sodium with norepinephrine storage might be a basic pathogenetic mechanism in hypertension (4).

Our finding of an increase in the efflux rate of norepinephrine from platelets in DOCA and salt treated rats and of a correlation between heart weight and efflux rate seem to strengthen the evidence that the storage of norepinephrine in neuronal cells varies with that in platelets, as long as platelet aggregation and the release reaction are prevented. In an earlier study it was shown that the release of norepinephrine and serotonin from platelets have different kinetics (11). A recent study shows that norepinephrine is released from α-granules by exocytosis (L. Stavenow, I. Mattiasson, B. Theodorsson and B. Hood. To be published).

The observations reported here indicate that platelets can serve as a valid model for the study of granular storage of norepinephrine. The way in which sodium interferes with the storage of norepinephrine is however still enigmatic.

Acknowledgements

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SPONTANEOUS EFFLUX OF PLATELET α-GRANULE COMPONENTS IN RELATION TO 14C-NOREPINEPHRINE IN BUFFERS.

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(Recived in final form March 22, 1982)

Summary

In an investigation of the spontaneous efflux of beta-thromboglobulin (β-TG), 14C-norepinephrine (14C-NE) and growth stimulating activity (GSA), measured as incorporation of 3H-thymidine in rabbit aortic smooth cells, from platelets resuspended in Ca^{++}- and Mg^{++}- free buffer a continuous increase in 3H-thymidine incorporation was seen during 120 min. There was good stoichiometric parallelism between β-TG and 14C-NE during the 80 minutes the experiment lasted, indicating that norepinephrine is incorporated in α-granules and released by exocytosis.

Introduction

Platelet derived growth factor (PDGF) is a cationic polypeptide that stimulates DNA-synthesis and cell replication in connective tissue cells in vitro (1). During aggregation PDGF, contained within platelet α-granules (2-3) is released with other α-granule components, i.e. beta-thromboglobulin (β-TG), platelet factor 4 and platelet fibrinogen (2). It has been suggested that PDGF may play a major role in the pathogenesis of atherosclerosis by inducing intimal proliferation at the site of endothelial injury (4). However, the biochemical mechanisms regulating the platelet release reaction are not properly understood. Recent studies have suggested that the platelet release reaction can proceed to a considerable degree largely independent of aggregation (5-8).

The present study is based on the observation by Mattiasson et al that the net efflux rate of norepinephrine (NE) from platelets, preincubated with 14C-NE and resuspended in buffer, varies with the diastolic blood pressure (9). A significantly faster net efflux of NE has been found in a group of normotensive first degree relatives of hypertensive individuals (10). These findings raise the question whether the efflux of NE is due to exocytosis or to a selective release from an intragranular binder. If the cause is exocytosis, known intragranular substances should be released with stoichiometrical constancy with NE.

The aim of the present investigation was to elucidate the spontaneous efflux of growth stimulating activity (GSA) and β-TG from platelets in relation to 14C-NE. The major part of the GSA from platelets is probably due to PDGF but the coexistence of other growth factors cannot be excluded.

Materials and methods

Experimental procedure

The efflux of NE, β-TG and GSA was studied with the method determinating efflux

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of 14C-NE described by Mattiasson et al (9). Blood was obtained from apparently healthy volunteers and was collected in 10 ml siliconized tubes, each containing 1 ml NaCl 0.12 M and EDTA 0.027 M. Within 2 hours of venipuncture the platelet-rich plasma (PRP) obtained from each individual was incubated with 14C-NE, (0.1 μCi/ml PRP=2.7 x 10^{-6} M) for 90 min at 37°C. Platelets were isolated by centrifugation (3000 g, 10 min) and resuspended in Krebs-Ringer bicarbonate buffer from which Ca^{++} had been removed. Aliquots were taken for determination of 14C-NE, β-TG and GSA immediately after resuspension (zero time) and thereafter at regular intervals for 80-120 minutes. Platelets were separated by centrifugation and determinations were made of 14C-NE, β- TG and GSA in the supernatant. 14C-NE activity was determined by liquid scintillation counting. Quench correction was made by external standard.

Determination of β-thromboglobulin

Purified β-TG was kindly provided by Dr Duncan Pepper, Blood Transfusion Service, Royal Infirmary, Edingburgh. Isolation of β-TG and the development of a radioimmunoassay for measuring this substance in body fluids have been described earlier (11, 12). Radioiodinated β-TG-tracer was prepared with the lactoperoxidase method (13). Protein bound I^{125} was separated from unbound I^{125} by chromatography on a Sephadex G 50 column with a bed dimension of 1 x 45 cm and was eluted in 1 ml fractions at a flow rate of 20 ml /hour with 0.075 M barbital buffer, pH 8.6, containing 0.01 M EDTA and 0.05% sodium azide. The column was pretreated with bovine serum albumin (BSA). The fractions were collected in polypropylene tubes containing 1 ml of 2% BSA in eluant buffer. The immune reactivity of labelled β-TG was measured from its reaction with excess anti- β-TG antibody. 90-95% of the purified tracer was bound. Antisera against purified β-TG were raised in rabbits. The diluent used in the assay was 0.075 M barbital buffer, pH 8.6 containing 0.5% BSA, 0.01 M EDTA and 0.5% sodium azide. To dilutions of unknown samples or of a standard β-TG solution (0, 12.5, 25, 50, 100 and 200 ug/l) was added I^{125}-β-TG to a final concentration of 1 μg/ml. Antiserum was then added to a final dilution of 1/160.000. The final incubation volume was 500 μl. The mixtures were incubated at 4°C overnight. Antibody-bound tracer was separated from the unbound tracer by a double antibody method described by Wood et al (14). Following the addition of 50 μl non immune rabbit serum (diluted 1/100) and 500 μl goat anti-rabbit antiserum (diluted 1/20) containing 5% polyethyleneglycol 6000 (PEG) the tubes were incubated for 1 hour at room temperature. The precipitate was collected by centrifugation at 5.000 g for 30 minutes at room temperature. The supernatant was aspirated and the radioactivity of the precipitate was measured in a 16 channel Nuclear Enterprise NE 1600 Counter to which an ABC 80 computer was connected. A typical standard curve is seen in figure 1.

Known amounts of purified β-TG were added to normal plasma to a final concentration of 12,5, 25, 50 and 100 μg/l. The recoveries were 105, 110, 109 and 110%, respectively.

During platelet incubation 600 μl samples were collected with the aid of polypropylene pipettes and transferred to polypropylene tubes containing 20 μl 0.01 m EDTA, 20 μl 2,8 uM prostaglandin E_1 (Sigma) and 20 μl 30 mM theophylline maintained at 0°C. After having been mixed the samples were immediately centrifuged at 2,300 g for 15 minutes at 4°C and two thirds of the supernatant was removed for assay.

Growth stimulating activity

GSA was calculated from the amount of 3H-thymidine incorporated in rabbit aortic smooth muscle cells (SMC). Rabbit (SMC) were grown from explants of medial

segments of the thoracic aorta from normal rabbits as described by Ledet (15). 8-10 explants were incubated in 24 cm^2 plastic flasks (NUNC A/S) containing 2 ml of Minimal Essential Medium (MEM) (GIBCO), HEPES buffered(20 mM), supplemented with 10% fetal bovine serum (GIBCO heat inactivated), L-glutamine 585 mg/l, non-essential amino acids (NEAA) and gentamicin 0.1 mg/ml. The flasks were incubated at 37°C and left untouched for 10 days for estabilish most of adhesion and primary growth. Growth was observed from approximatively 80% of the explants after 7 days. Medium was changed twice a week. Subcultures were obtained by the use of 0.05% trypsin - 0.02% EDTA. Confluent cells from passages 2-4 were used for the experiments.

3 ml of a suspension of trypsinized cells was transferred to 10 cm^2 plastic Petri dishes (NUNC) and the cells were allowed to settle. The cell layer was washed twice with 2 ml Dulbecco phosphate buffered saline (PBS) (FLOW) and 3 ml serum-free MEM containing the above mentioned supplements was added. The cells were left 24 hours to equilibrate. 300 ul of the supernatant was added to each dish and incubation was continued for 24 hours. 1 uCi/ml medium of (methyl-3H)-thymidine (Amersham) was added to each dish after 18 hours' of the incubation. The 3H activity was determined as described by Witte et al. (2), but with 10 ml Aquasol 2 (New England Nuclear) as scintillation fluid. All cell culture experiments were performed in triplicate.

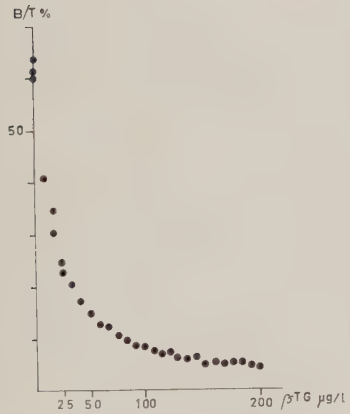

Fig. 1

B/T (bound counts/total counts x 100) versus concentration of human β-thromboglobulin. The concentration of liquid is given as the concentration of the standard solution (of which 50 µl is added to each tube; the volume of each sample is also 50 µl). Only values within the range of 10-100 µg/l were accepted. Within this range logit-log transformation gave a completely straight line. If more than one dilution of a sample could be accepted, the mean value was used.

Determination of Lactic Dehydrogenase

Lactic Dehydrogenase (LD) was measured by following the decrease in NADH in a system containing pyruvate (16).

Measurement of platelet aggregation

Aliquots were taken for measurement of 14C-NE efflux and changes in light transmission in an aggregometer (Paton Dual Chanel Aggregometer). Aggregation in two of the aliquots was measured at 37°C during continous stirring and recording of light transmission for 80 min. Purified bovine thrombin (0.5, 1 and 3 U/ml) was added to one of the aliquots at 30 minutes.

Results

There was a continous efflux of GSA into the buffers during the 120 minutes

the experiments lasted. GSA-efflux from platelets from 17 subjects was measured 25 times and the results were always similar (figure 2).

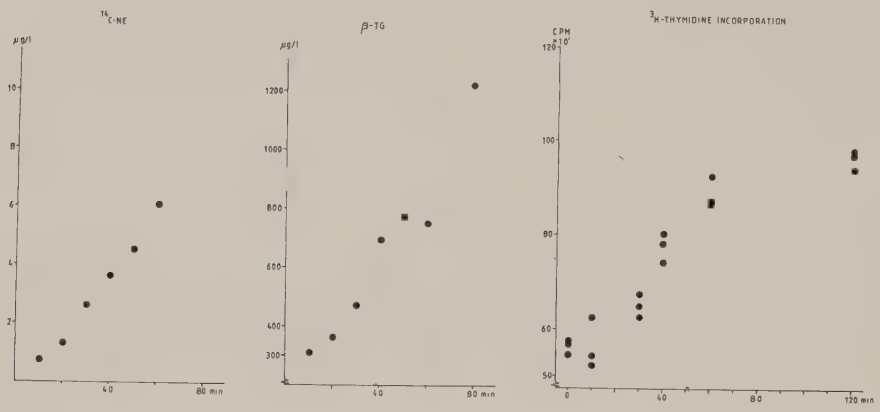

Fig. 2

Time dependent efflux of 14C-norepinephrine, β-thromboglobulin and growth stimulating activity in a typical experiment. Determinations were made in three aliquots of a platelet rich plasma from one individual.

GSA activity was measured in parallel with β-TG and 14C-NE in 6 individuals. The slope of the curve varied more than that of the curves for β-TG and NE. The difference was most likely due to the use of different cell populations for the GSA assay (figure 2).

Values for 14C-NE were corrected for background radioactivity by subtracting zero time values. To make β-TG measurements comparable with 14C-NE measurements the same correction was made for β-TG values. Measurements were made in incubates from 10 individuals, in two of them twice repeated at two different occasions. The quotient 14C-NE/β-TG in each platelet incubate showed good stability without any systematic deviation in either direction (figure 3).

In the calculations 35,800 was used as the molar weight of β-TG (17). The dispersion in quotients between different individuals was substantial, ranging from 0.28 to 3.81. However, in the two individuals where measurements were made on two different occasions, both quotients were largely the same, one individual having a low quotient and the other a high one at both occasions. This might indicate interindividual variation either of the ability to take up or bind NE in granules or of the amount of β-TG per granule. The release of LD was very small, 0-9% of the total LD content, and not parallel to efflux of NE or β-TG. No change in light transmission was seen in the aggregometer during 80 min, although the release of 14C-NE was continuous and in the same range as usual (figure 4).

Discussion

The spontaneous efflux of GSA, β-TG and NE in Ca^{++}- and Mg^{++}- free buffer was studied. A parallelism was found between the effluxes of NE and β-TG. Also GSA

showed a similar efflux, although the correlation was not striking, probably because of varying density and ages of the cells in the culture. But there was a very definite efflux of GSA. The efflux was allowed to proceed several hours and within 24 hours of spontaneous efflux we could usually demonstrate β-TG and GSA differing substantially from those noted after thrombin treatment (to be published). Similar observations have been made by McCann and Hagen (7) who found that when added to SMC in culture the stimulating capacity of a platelet suspension of intact platelets was more than twice that of thrombin-aggregated supernatant.

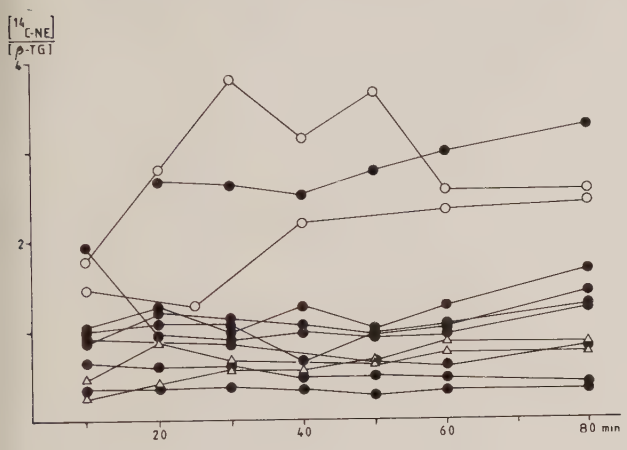

Fig. 3

Quotient of β-thromboglobulin (mol/l) in 10 individuals during 80 min spontaneous efflux. In two individuals (○) and (△) determinations were made on two separate occasions.

The mechanisms of the efflux studied are not properly understood. We tried to minimize aggregation by not include Ca^{++} in the buffers. No aggregation could be detected in a Born aggregometer during the experiments (fig 4). The kinetics of α-granules and dense bodies are different, as shown by Witte et al. (2). Since there was little or no release of lactic dehydrogenase cell lysis seems very unlikely. Furthermore the release of serotonin has been shown to be slower than that of NE (9). These findings which have been confirmed by Pletscher (19) argue strongly against cell lysis being able to explain the NE-release.

Platelet hyperfunction can be measured in different ways. Platelet aggregation in response to known stimuli, such as collagen, thrombin and ADP, as well as platelet survival studies are established techniques (19,20). Plasma levels of platelet specific proteins, such as β-TG are regarded as the best markers of the in vivo platelet release reaction (19). Which aspect of platelet function is most relevant in atherogenesis is not clear. In vivo release of β-TG should vary with the release of PDGF, and high plasma levels of β-TG might reflect increased influence of proliferative stimuli on the arterial wall.

Whether there is a spontaneous efflux of α-granule proteins in vivo cannot be decided from our experiments. However, some studies on record lend support to such a possibility. O'Brien (5) has suggested that platelets continuously release their granules while in the circulation, and the finding that platelets that have undergone the release reaction continue to circulate may further support this hypothesis. Bolton et al. (6) found a reduction in β-TG content of platelets in patients with leukemia treated with chemotherapeutics which inhibited platelet production and concluded that β-TG leaks from, or is actively secreted by, platelets in the circulation. In other studies neither treatment with aspirin nor long-term infusions of prostacyclin, has been found to have any effect on plasma β-TG or platelet factor 4 despite a marked decrease in platelet aggregation (8, 21). Own studies indicate that aspirin has no effect

and prostacyclin only a slight initial effect on the efflux rate of NE and β-TG in our system (manuscript in preparation).

We found that for 60 minutes after resuspension in buffer, i.e. the duration of our earlier studies of NE-efflux, NE and β-TG were secreted with stoichiometrical constancy. The most reasonable explanation would then be that in the individuals studied in the present investigation NE was secreted from α-granules by exocytosis. But it cannot be excluded that NE and β-TG are excreted at the same rate from two different pools. However, this seems less likely, especially as the release of serotonin is definitely slower. The higher efflux rate of NE in hypertensive individuals (9) should then also reflect a more rapid efflux of GSA. This assumption is strengthened by Fischer-Dzoga's (22) observation that the lipid-free bottom fraction of serum from hypertensive monkeys has an increased proliferative effect on SMC in culture.

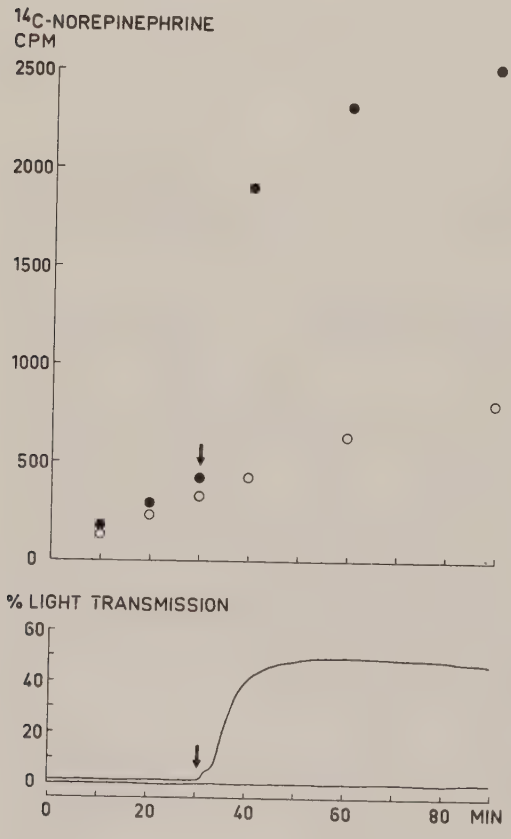

Fig. 4
Effect on 14C-norepinephrine and change in light transmission during 80 min in two aliquots of platelets suspended in Ca^{++} free buffer. To one aliquot (upper curves) thrombin (1 U/ml) was added at 30 min. (arrow).

Literature studies and the finding in the present investigation appear to warrant the conclusion that there is some evidence that the platelet release reaction can proceed spontaneously even in the absence of considerable aggregation. Though it is too early to speculate about the significance of a possible spontaneous platelet release reaction in vivo, a continuously secretion by

platelets might lead to long-lasting stimulation by PDGF and/or other growth stimulating factors on the arterial wall. This could be of importance in the pathogenesis of atherosclerosis, especially when the permeability of the endothelium is for some reason increased.

Acknowledgements

We thank miss Barbro Christensson for skilful technical assistance. The study was supported by grants from the Ernhold Lundström Foundation and The Swedish National Association against Heart and Chest Diseases.

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EFFECTS OF THROMBIN, ACETYL SALICYLIC ACID AND PROSTAGLANDINS ON SPONTANEOUS EFFLUX OF NOREPINEPHRINE AND BETA-THROMBOGLOBULIN FROM PLATELETS IN CALCIUM/MAGNESIUM DEFICIENT ENVIRONMENT

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ABSTRACT

The effect of different substances on the spontaneous efflux of 14C-norepinephrine (14C-NE) and beta-thromboglobulin (βTG) from platelets in Ca^{2+} - and Mg^{2+} - free buffer was tested. Addition of increasing concentrations of thrombin (0.015-0.625 U/ml) resulted in an immediate increase of 14C-NE detectable in the supernatants, but the slopes of the efflux curves from 10 min after thrombin addition were almost identical and parallel with the slope before thrombin addition. Also beta-thromboglobulin was steadily released efter thrombin addition. Prostaglandin E_1 and prostacyclin in concentrations that gave a 30-fold increase in cAMP accumulation resulted in a moderate decrease in efflux of 14C-NE from 10 to 30 min but did not influence the efflux thereafter up to 80 min. Acetyl salicylic acid in vivo and in vitro (200-500 µmol/l) did not influence the efflux of 14C-NE or βTG. The conclusions are that factors known to exert considerable effects on aggregation, desaggregation and acute release produce only transitory effects in a Ca^{2+} - and Mg^{2+} - free environment and that the basal efflux of α-granule components seems to be a stable process.

INTRODUCTION

Spontaneous release of 14C-norepinephrine (14C-NE) from the platelets of normo- and hypertensive subjects to a Ca^{2+} - and Mg^{2+} - free buffer has previously been studied. These platelets had been preloaded with the labeled norepinephrine for 90 min in plasma and a positive correlation between the rate coefficient of initial efflux and the blood pressure level was found

Key Words: α-granule components, platelet efflux, thrombin, acetyl salicylic acid, prostaglandins.

(1). An overrepresentation of high efflux rates in normotensive relatives to hypertensive individuals was also found (2). A continuous efflux of cell growth stimulating activity for the arterial myocytes and beta-thromboglobulin (βTG) at the same rate as 14C-NE could also be demonstrated (3). These findings suggested that the spontaneous release of 14C-norepinephrine could be attributed to exocytosis of alpha-granules. The spontaneous release of serotonin occurred more slowly and independently of the release of norepinephrine (1). The aim of the present investigation was to study the influence of low concentrations of an aggregating substance, thrombin, and the inhibiting substances acetyl salicylic acid, prostaglandin E_1 and prostacyclin on the spontaneous release of 14C-NE from platelets to further elucidate the nature of this process.

The platelet inhibitory substances were chosen because of their different mechanisms of action on the platelet. Acetyl salicylic acid inhibits cyclooxygenase and thereby thromboxane synthesis. Prostaglandin E_1 and prostacyclin stimulate adenylate cyclase leading to increased cyclic AMP (cAMP) levels. This nucleotide probably inhibits platelet aggregation by decreasing calcium concentration in the platelet cytosol (4, 5).

MATERIAL AND METHODS

The efflux of norepinephrine was studied as previously described (1, 2). Platelet rich plasma (PRP) for the thrombin experiments was obtained from the blood bank. Blood was collected in polyethylene bags containing CDP-adenine (citric acid 0.33 g, sodium citrate 2.6 g, sodium phosphate 0.22 g, glucose 2.32 g, adenine 27.5 mg/100 ml) and centrifuged at 3400xg for 7.5 min. The platelet concentration was adjusted to about 250×10^9/l with platelet poor plasma. For other experiments blood was collected from healthy individuals in 10 ml siliconized vacutainer tubes containing 1 ml NaCl 0.12 mol/l and Na_2EDTA 27 mmol/l. PRP was obtained by centrifugation (120 x g, 15 min) and was kept at room temperature until the experiment started within 2 hours. PRP was incubated with 14C-NE [DL (carbinol - 14C) noradrenaline DL bitratrate, 55 mCi/mmol Amersham] to a final concentration of 1.8×10^{-6} mol/l (= 0.1 μCi/ml PRP) for 90 min at 37°C and with continuous slow shaking. The incubation was interrupted by cooling on ice. Platelets were isolated by centrifugation (3000 x g, 10 min, 4°C) and resuspended in Krebs-Ringer bicarbonate buffer (pH 7.4) free from calcium and magnesium. Immediately after resuspension aliquots (0.5 ml) were taken for determination of uptake and zero-time values. The resuspended platelets were again placed in the water bath. Aliquots were taken at regular time intervals up to 120 min. Platelets were separated by centrifugation (10000 x g, 10 min) and radioactivity was measured in the supernatant. For uptake values radioactivity was measured in the pellet after sonication. The value was corrected for platelet count made in duplicate in a celloscope. Bovine thrombin purified according to Lundblad et al (6) was kindly provided by Björn Dahlbäck, Dept. of Clinical Chemistry, Malmö. It was added in concentrations of 0.015 - 0.625 U/ml PRP either immediately after the platelet resuspension was placed in the water bath (zero time) or at 30 min. In some series of experiments with thrombin platelets were preloaded with varying concentrations of 14C-NE (0.06 - 0.2 μCi/ml).

Acetyl salicylic acid was added at zero time to a final concentration of 200 and 500 μmol/l. In vivo effect was tested by giving a daily dose of 0.5 g easily soluble acetyl salicylic acid for 4-7 days before sample collection. Prostaglandin E_1 (Sigma) was used in a final concentration of 5×10^{-6} mol/l and was added both at start of incubation with 14C-NE and at zero time or only at zero time. Prostacyclin (kindly provided by Dr. A.J. Salter,

The Wellcome Research Laboratories) was added to a final concentration of 2.5×10^{-7} mol/l at zero time or at 0, 5, 10 and 20 min.

The total amount of cAMP in platelets and incubation medium was determined according to a prelabeling technique in which 3H-adenine was used for the subsequent determination of 3H-cAMP (7). 100 µl adenine (10 µCi 3H-adenine, Amersham) was added to 900 µl PRP and was incubated for 60 min at 37°C with continuous shaking. Platelets were separated by centrifugation (3000 x g, 10 min, 4°C). The supernatant was decanted and the tubes were wiped carefully. Platelets were resuspended in one ml of Krebs-Ringer buffer and 40 µl of either prostaglandin E_1 or prostacyclin were added. Reaction was stopped after 10 min by adding 100 µl 2% sodium dodecyl sulfate and the tube was placed on ice. Platelets were sonicated (Branson Sonic Power Comp B12, 15 sec. at setting 6). 50 µl 14C-cAMP was added for recovery calculation. 3H-cAMP was determined chromatographically according to Salomon et al (8). Results are expressed as disintegrations per minute (dpm) per 10^9 platelets.

Beta-thromboglobulin (βTG) was determined radioimmunologically according to a method earlier described (3). Purified βTG was kindly provided by Dr. Bo Theodorsson, Coagulation Laboratory, Malmö.
Wilcoxon's test was used for the statistical calculations.

RESULTS

Effects of Thrombin. Addition of increasing concentrations of thrombin resulted in an increase in 14C-NE detectable in the supernatants. The slopes of the efflux curve were almost identical for all thrombin concentrations (0.035-0.625 U/ml) in reproducible experiments (Fig 1).

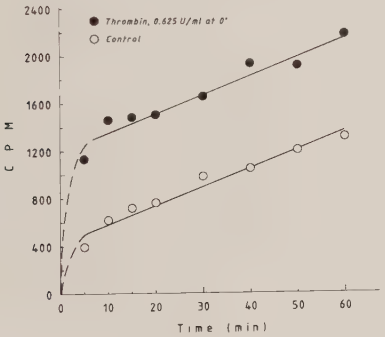

Fig. 1

Efflux of 14C-norepinephrine under basal conditions (○) and after the addition of thrombin (0.625 U/ml) at 0 minutes (●) in aliquots.

When thrombin was added after 30 min the slope after thrombin addition was parallel with the initial slope (Fig 2).
When platelets had been preloaded with a higher concentration of 14C-NE (0.2 µCi/ml) the linear slope of the 14C-NE efflux was greater as was the increment of increased efflux upon thrombin addition than for platelets loaded with the standard or lower dose of 14C-NE. The slope of the efflux curve was, however, nearly identical to the control curve in all cases.
In five experiments βTG release was measured under basal conditions and after addition of 0.5-3.0 U/ml of thrombin. The simultaneous efflux of βTG was not influenced by 14C-NE preloading values in the control curves. After 10 min a pronounced release of βTG was found comprising about 25-35% of the total content of βTG in the platelets. The release of NE was close to 50% of

total NE content in all experiments. In most experiments there was a decrease in βTG value after thrombin addition, followed by another increase. This may have been an effect of thrombin itself on βTG or of the hydrolytic enzymes released after the action of thrombin. Therefore platelets were separated from the buffer by centrifugation 5 min after thrombin addition, resuspended in fresh buffer and the spontaneous release of βTG at 37°C was again studied as initially. We then found a continuous steady release of βTG. (Fig 3). The decreased slope of efflux curve after resuspension could be ascribed to the loss of platelets in this second centrifugation. To test the influence of thrombin on βTG 0.625 u/ml of thrombin was added to purified βTG

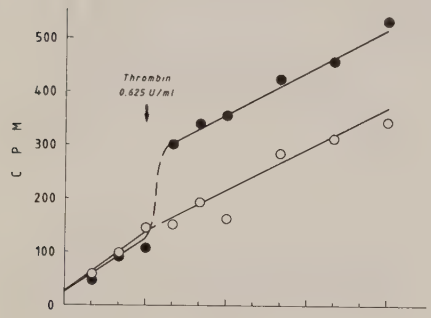

Fig 2.

The effect of thrombin on the efflux of 14C-NE from platelets preloaded with 0.1 μCi/ml. Thrombin was added at 30 min to a standard concentration of 0,625 U/ml (●).

in a concentration corresponding to the concentration of βTG in platelets. No effect on measured βTG was seen. However, sonication of purified βTG reduced the concentrations with 30-40%.

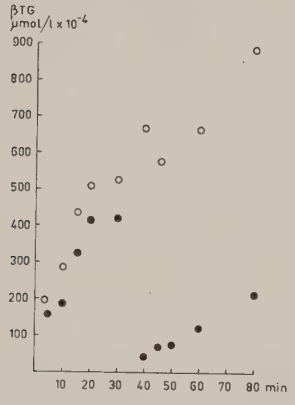

Fig 3.

Efflux of β-thromboglobulin in two aliquots. To one (●) thrombin (0.5 U/ml) was added at 30 min, the platelets were separated by centrifugation after 5 min and were resuspended in fresh buffer.

Effects of Prostaglandin E_1 and Prostacyclin. Prostaglandin E_1 at the concentration of 5×10^{-6} mol/l gave a marked effect on cAMP accumulation which increased 30 times (Fig 4). Prostacyclin at 2.5×10^{-7} mol/l gave similar effects increasing the accumulation of 3H-cAMP from 18 to 481 dpm/10^9 platelets when assayed at 10 minutes. When prostaglandin E_1 was added at zero time (n=8) the initial slope of the linear efflux curve

(5-20 min) showed a moderate decrease in all but one experiment ($p<0.05$). The slope in the 30-80 min interval was not affected (Fig 5). When prostaglandin E_1 was added both at the start of incubation with 14C-NE and at zero time for the release studies (n=8) the decrease was more pronounced. However, in these experiments a lowering of 14C-NE uptake was noticed, which could explain the difference. The effect of prostacyclin was almost identical with that of prostaglandin E_1, both when it was added only once (n=4) and at repeated intervals (n=4) (Fig 6). It was obvious that with both prostaglandin E_1 and prostacyclin the effect on the efflux rate was very pronounced in some individuals and rather slight in others. Particularly in the prostacyclin experiments, the greatest inhibition seems to occur in those with the highest spontaneous release. The effect on cAMP was the same with the doses of prostaglandin E_1 and prostacyclin used. If, for cal-

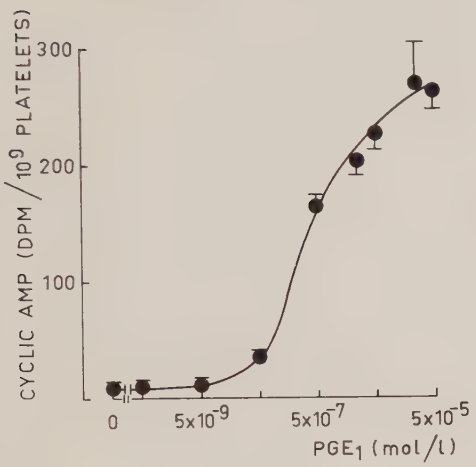

Fig 4.

The effect of prostaglandin E_1 on accumulation of 3H-cAMP in platelets. Each point represents the mean ±SD of three determinations.

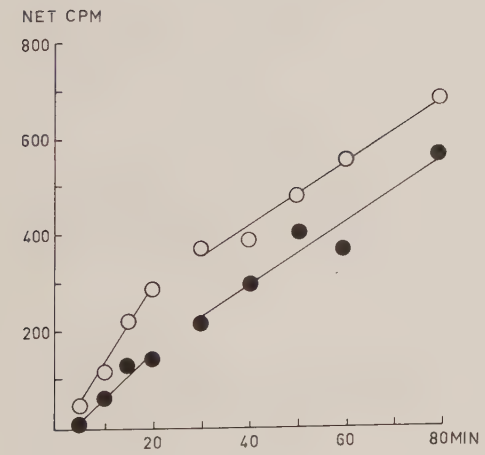

Fig 5.

The effect of prostaglandin E_1 ($5x10^{-6}$ mol/l) on efflux of 14C-norepinephrine (●) compared to a control (○) in a typical experiment.

culation, the two series of prostaglandin E_1 and prostacyclin are put together the decrease in initial efflux was highly significant ($p<0.01$), but the slope between 30 and 80 min did not differ from the controls.

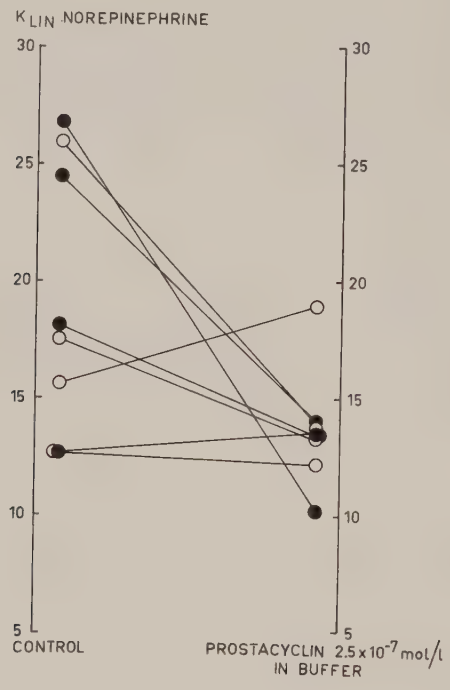

Fig 6.

Linear regression coefficient for efflux of 14C-norepinephrine during 5 to 20 minutes before and after addition of prostacyclin (2.5×10^{-7} mol/l) at 0 min (○) and at repeated doses (●).

Effects of Acetyl Salicylic Acid: In vitro: In previous studies on norepinephrine efflux from platelets we have used the regression coefficient for the semilogarithmic efflux curve (k) to enable comparison of non-equal samples, thereby minimizing the influence of variable platelet count and preloading (1). No significant effect on k for the efflux of 14C-NE was seen when acetyl salicylic acid was added in the concentrations of 200 and 500 µmol/l. In this study aliquots of the same preparation were used which made it possible to compare the initial linear slope (10-30 min). No significant difference was seen after addition of acetyl salicylic acid.
In vivo: In this part of the investigation the simultaneous release of βTG and 14C-NE was studied. The effect of acetyl salicylic acid was tested in four healthy males taking 0.5 g aspirin for 4-7 days. In three individuals there was no difference in the k values. In one subject a slight increase in both βTG and NE efflux was seen. This suggests that acetyl salicylic acid in vivo has little effect on the spontaneous efflux of alpha granule components.

DISCUSSION

In an earlier report we have described the spontaneous release of alpha granule components and 14C-NE from platelets resuspended in buffers (3). There was no change in light transmission in the Born aggregometer although

the efflux of NE was substantial. By simultaneous analysis of lactic dehydrogenase cell lysis could be excluded as an explanation for the spontaneous release. The existence of a spontaneous platelet release in vivo remains to be demonstrated, but there are some indirect indications of such a reaction in vivo (9, 10). In the present study we have tried to further evaluate the nature of the spontaneous release of NE and the simultaneous and parallel release of α-granule components seen in vitro in earlier studies. It is obvious that even small doses of thrombin provoke an immediate release of 14C-NE. The effect is, however, very transient and the spontaneous efflux after about 6-8 minutes returns to its original slope. The concentration of thrombin has no influence on the efflux rate after the initial thrombin treatment. There was a striking stability of the basal efflux process even after rather high thrombin doses. Even βTG showed a continuous efflux after thrombin treatment if platelets were separated from the media after thrombin addition and resuspended in fresh buffer. If platelets remained in the same buffer after thrombin treatment βTG-measurements showed a wide scatter with a tendency towards an immediate increase, a decrease and again an increase. This may be ascribed to the influence of hydrolytic enzymes released by thrombin on βTG, making βTG-determinations after thrombin treatment unreliable. The observation that the immediate release of βTG was lower than the release of NE (25-35% vs 50%) may indicate an underestimation of βTG-values. Sonication did decrease the measured βTG and furthermore hydrolytic enzymes are released at sonication. Another explanation could be that only some of the α-granules had been preloaded with NE, with a preference for those lying close to the plasma membrane also being most subjected to exocytosis at thrombin treatment.

The concentration of acetyl salicylic acid used in the in vitro experiment has been reported to block the synthesis of thromboxane A_2 almost completely (11). The aspirin doses given per os have been reported to decrease the release induced by ADP, epinephrine and arachidonic acid by up to 99% (12). These results suggest that the spontaneous alpha granule release in vitro is independent of thromboxane A_2. The doses of prostaglandin E_1 and prostacyclin used here gave a 30 fold increase in cAMP concentration. In spite of this pronounced effect on cAMP the influence on the spontaneous alpha granule release was rather slight and transitory. However, there was an obvious effect, which was not seen when thromboxane synthesis was blocked.

Factors known to exert considerable effect on aggregation, desaggregation and acute release produce only transitory effects in a calcium and magnesium free environment. This applies even at levels which produce dramatic effects, e.g. thrombin at high concentrations. Although the basal efflux rate of α-granule components may be temporarily altered, it seems to be a stable process which reasserts itself after a short time.

ACKNOWLEDGEMENTS

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EFFLUX OF NOREPINEPHRINE FROM RAT PLATELETS, SYNAPTOSOMES AND HEART TISSUE.

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Summary

Platelets have the ability to take up, store and release biogenic amines and have therefore been used as models for neurons in studies of neuropsychiatric disorders. We have previously found an increased release of isotope labeled norepinephrine (NE) from platelets of hypertensive patients and in a group of normotensive subjects belonging to families with an accumulation of hypertension. In an attempt to validate our model we have compared the efflux of ^{3}H-NE from rat platelets, synaptosomes and heart tissue. The samples were all taken from the same animal. We have found that efflux from platelets correlates positively with efflux from synaptosomes as well as from heart tissue. This strengthens the hypothesis of increased spontaneous release of NE from sympathetic neurons in hypertension.

Introduction

For obvious reasons there is a lack of biochemical and physiological studies on the human nervous system in many neurological and psychiatric disorders. This has raised the question of whether or not platelets can be used as models of neurons. The concept is based on the ability of platelets to take up biogenic amines, store them in granules and finally release them.

Various amines are taken up by passive diffusion (1), but serotonin and norepinephrine (NE), at least, are transported by carrier mediated mechanisms (2). Serotonin is the most effectively taken up of all biogenic amines, but NE is concentrated in platelets as well, 500 times the plasma concentration (2). Within the platelet catecholamines are further concentrated and stored in granules that seem to have certain similarities with the storage granules of adrenal medulla and neuronal cells (For review see ref 3). Catecholamines can be released from platelets by the same mechanisms as in neurons. These are as follows: 1) by exocytosis, a process whereby the whole granular content is discarded, 2) by reserpine-like drugs which interfere with the transport systems in the granular membranes, and 3) by tyramine, which displaces the intracellular amines from their storage sites (For review see ref 4). Platelets, however, do not synthesize catecholamines. No activity of tryptophan hydroxylase (5) or dopamine-ß-hydroxylase (6) has been found. In spite of these limitations the model hypothesis has attracted several investigators:

Platelets of patients with Parkinson's disease have been found to take up less ^{14}C-dopamine than age-matched controls (7). Reduced levels of serotonin in platelets have been reported in Down's syndrome (for review see ref 8). Low serotonin uptake is found in patients with endogenic depression (9). We have earlier reported a more rapid spontaneous release of ^{14}C-NE from platelets of hypertensive patients and also a significant correlation between the individual NE-

efflux rate coefficient and the diastolic blood pressure (10). This increased release does not appear to be a secondary change due to the high blood pressure per se, as it is also found in a group of young normotensive relatives of hypertensive patients (11). DOCA and salt treatment in rats have earlier been demonstrated to induce a decreased storage of NE in tissue with a high degree of sympathetic innervation (12). The same treatment also induced an increase in the efflux rate coefficient for ^{14}C-NE from platelets (13), giving an indirect indication of a parallelism between the storage of NE in neuronal tissue and platelets. The aim of the present study is to further evaluate the platelet as a model of neuronal cells by direct measurement of the ^{3}H-NE efflux from preloaded synaptosomes, heart tissue and platelets from the same animal.

Material and methods.

Platelet rich plasma (PRP), synaptosomes and heart slices were prepared from 15 male and 15 female Sprague-Dawley rats, weighing between 250 and 300 g. It was always possible to obtain sufficient material to run duplicates of synaptosomes. However, due to the small plasma volume it was not always possible to get duplicates from platelets. In some preparations uptake values were very low, indicating a low number of platelets. Only rats where duplicates from platelets were obtainable or uptake values were >500 cpm were accepted. One aspect of this experiment was to study the effect of ethanol on NE-release. Twenty-one of 30 animals were therefore given a 24% ethanol solution as their only drinking fluid for 52-58 weeks, while the rest were given tap water. Results concerning the comparison of NE efflux between the two groups will be published elsewhere.

Synaptosomes-preparation and ^{3}H-norepinephrine-efflux

Synaptosomes were prepared according to Cotman (14). Rats were killed by decapitation and the brain was immediately taken out and placed in ice cooled 0.32 M sucrose solution. Rat cortex was homogenized in 10 volumes of ice cooled 0.32 M sucrose. A glass homogenizer with motor-driven teflon pestle was used and operated at 400 RPM with 8 stokes during one min. The homogenate was centrifuged at 1000xg for 10 min. The supernatant was then centrifuged at 12,000xg for 10 min, the pellet was resuspended in 3.5 ml 0.32 M sucrose and layered on a discontinuous gradient of 4%, 6% and 13% Ficoll in 0.32 M sucrose, 4 ml of each concentration. Each gradient was loaded with material from one rat brain. The gradients were centrifuged at 63,000xg for 45 min. Synaptosomes were collected at the interface of the 6% and 13% Ficoll layers.

1 ml of the synaptosome suspension was added to 4 ml of Krebs-Ringer buffer (118.5 M NaCl, 4.75 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.20 mM $MgSO_4$, 26.5 mM HEPES (sodium salt), 0.125 mM Pargyline, 11 mM Glucose, 0.134 mM EDTA and 1.14 mM Ascorbic acid pH 7.2). ^{3}H-NE (DL-[7-^{3}H]noradrenaline hydrochloride 16 Ci/mmol Amersham) was added to a final concentration of 4×10^{-8} M (3.23 µCi) and the synaptosomes were incubated at 37°C for 30 min in a shaking water bath. After centrifugation at 12,000xg for 10 min the pellet was washed by resuspension in 5 ml Krebs-Ringer buffer and centrifugation at 12,000xg for 10 min. The final pellet was resuspended in 5 ml Krebs-Ringer buffer and kept on ice until the efflux study started. Efflux of NE was studied by incubation of synaptosomes in a shaking water bath at 37°C. Aliquots (0.5 ml) were taken at 0, 5, 10, 15 and 20 min, and kept on ice until they were centrifugated at 12,000 g for 10 min. The supernatant was transferred to scintillation vials containing 10 ml scintillation fluid and radioactivity was determined by liquid scintillation counting. Uptake of ^{3}H-NE was estimated by measuring 0.5 ml of synaptosomal suspension at time 0. For determination of uptake curves aliquots were taken at regular time intervals for 50-120 min during the incubation period. All determinations were made in duplicate. Counts obtained were corrected for background activity by subtracting the zero min value.

Platelet-preparation and ^{3}H-norepinephrine efflux.
Blood was collected in a plastic beaker containing 1 ml of 1% EDTA in 0.7% NaCl. PRP was prepared within one hour by centrifugating the blood two times at 120xg for 15 min. Duplicates of 0.8 ml PRP from every rat were taken, to which ^{3}H-NE was added to a final concentration of $5x10^{-7}$ M (6.4 uCi). Samples were then incubated for 90 min at 37°C with continuous slow shaking. Incubation was stopped by centrifugation at 3000xg for 10 min at 4°C. The pellet was washed by resuspension in 1.6 ml of Krebs-Ringer buffer (122 mM NaCl, 4.0 mM KCl, 25 mM HEPES and 1.23 mM KH_2PO_4, pH 7.4) and centrifugation at 3000xg for 10 min at 4°C. The final pellet was resuspended in 0.8 ml Krebs-Ringer buffer and incubated at 37°C with continuous slow shaking. Aliquots (0.1 ml) were removed at 0, 5, 10, 15 and 20 min. Tubes were kept on ice until centrifugation at 12,000xg for 10 min. 80 µl of the supernatant was transferred to 10 ml scintillation fluid. Uptake of ^{3}H-NE was determined by transferring 0.1 ml PRP-suspension at time 0 directly to the scintillation fluid. For determination of the uptake curve aliquots were taken at regular time intervals for 140-300 min during the incubation period. Radioactivity was determined as described above; counts obtained were corrected for background activity by subtracting the zero min value.

Heart-preparation and ^{3}H-norepinephrine-efflux.
NE-efflux form heart tissue was studied in a subsample of 16 rats (M=6, Fe=10). Hearts were taken out immediately after decapitation of the rats and kept in ice cooled saline during preparation. Thin slices were made with a scalpel and about 60 mg of heart tissue was added to 3 ml Krebs-Ringer buffer (the same composition as in the synaptosomal experiment). ^{3}H-NE was added to a final concentration of $5.75x10^{-7}$M (30 uCi) and the samples were incubated at 37°C for 3 hours. After washing in 3x3 ml buffer, 5 ml of buffer was added and efflux was studied at 37°C in a shaking water bath. Aliquots of 50 ul were taken at 0, 2.5, 5, 10, 15 and 20 min and were transferred directly to scintillation fluid as above. All determinations were made in duplicate and were corrected for background activity as above.

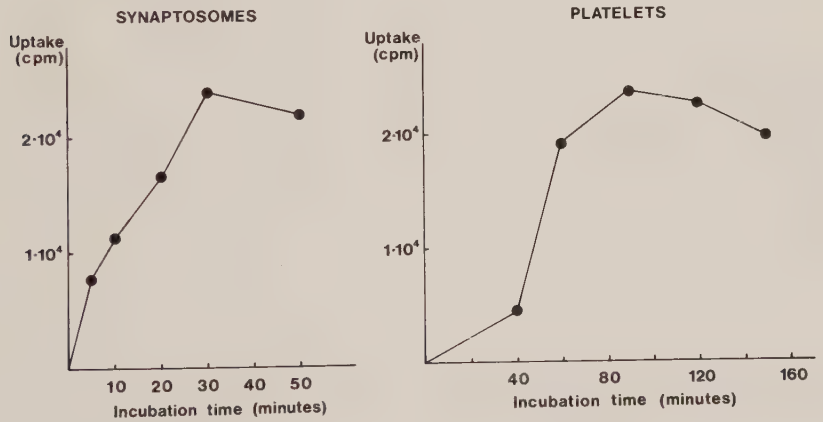

Fig 1.

Uptake of ^{3}H-norepinephrine in rat synaptosomes and platelets. A typical experiment.

Results.

The time dependent ^{3}H-NE uptake in synaptosomes and platelets is shown in Fig 1. The uptake curve of synaptosomes reaches a plateau much earlier than that of platelets, which explains our use of different incubation times for synaptosomes and platelets, 30 and 90 min respectively. For heart tissue we have arbitrarily chosen an uptake time of 3 hours, since the sympathetic tissue in this preparation is contained within a large amount of smooth muscle fibers and thus a slower uptake process can be expected.

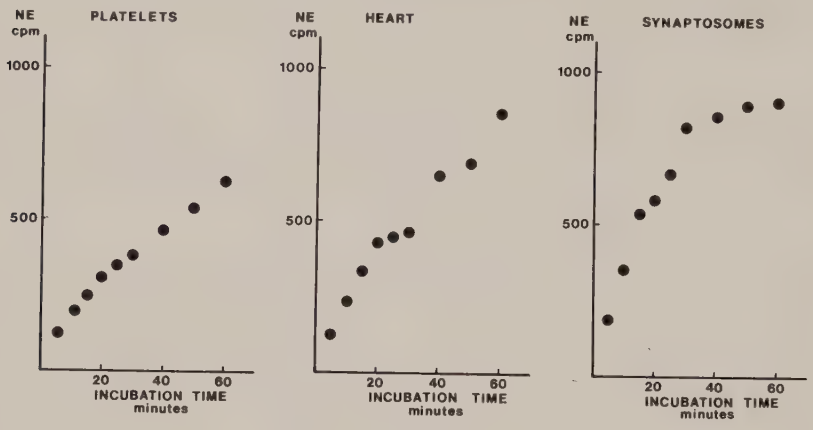

Fig 2.

A typical example of ^{3}H-norepinephrine efflux from platelets, heart tissue and synaptosomes.

The correlation between protein concentration and uptake value in the synaptosomal preparations was 0.81 ($p<0.001$), indicating a constant purity of the preparations. Counts obtained from the efflux studies were plotted against time and a linear relationship was found up to 20 min in all three systems (Fig 2). Only curves with a coefficient of correlation >0.9 were accepted. By linear regression the slope of the line was calculated using the formula:

$$\text{slope value} = \frac{\Sigma xy - \frac{1}{N}(\Sigma x)(\Sigma y)}{\Sigma x^2 - \frac{1}{N}(\Sigma x)^2}$$

where x is time in minutes and y is CPM measured.

There was a highly significant linear relationship between this calculated slope value and uptake values for platelets and synaptosomes ($r=0.74$ and $r=0.79$ respectively). In the heart preparation, no significant correlation was found between the slope values and the uptake, but there was a significant correlation between slope value and the weight of the heart slices ($r=0.53$). The slope value was divided by the uptake values for synaptosomes and platelets and multiplied with the mean uptake value for the preparations. The slope value from heart tissue was divided by mg of weight and multiplied with the mean weight. The quotientes were used as expressions of efflux rate and designated K_{lin}. There was no correlation between the uptake values in synaptosomes and plate-

lets.
We found a highly significant positive correlation between K_{lin} in platelets and synaptosomes ($r=0.67$, $p<0.001$) (Fig.3). There was also a positive correlation between K_{lin} in platelets and heart tissue ($r=0.55$, $p<0.05$) (Fig. 4).

Discussion.

In this study we have modified our previous method for NE-efflux in platelets. In earlier studies, ^{14}C-NE was used and a considerable amount of PRP was needed requiring pooling of plasma from several animals (13). Use of ^{3}H-NE with a higher specific activity allows us to take small amounts of rat PRP and to make individual comparisons between platelets and synaptosomes or heart tissue. In previous studies, NE-efflux in platelets was studied for a period up to 60 min and the efflux curve was well fitted to an exponential equation. Due to the more rapid efflux process in synaptosomes and heart tissue (Fig. 2), we have limited our study of the efflux process to the initial 20 min in the platelet experiment as well. With that time limit, the efflux curve is well fitted to a straight line in all three cell systems.

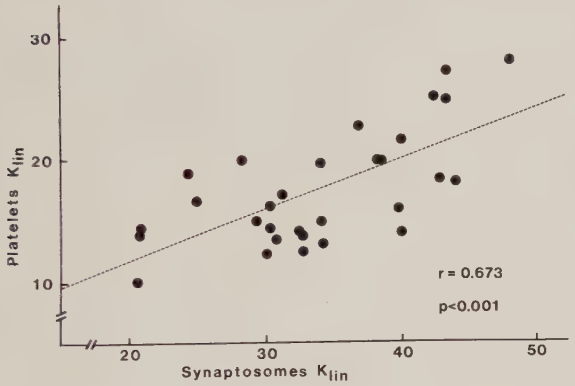

Fig 3.

Correlation between rate constant of norepinephrine efflux, K_{lin} (min^{-1}), from rat platelets and synaptosomes (n=30).

For the comparison of NE-efflux in three different cell systems in vitro different techniques of preparation and buffer composition have been used. For instance, Ca^{2+} and Mg^{2+} were omitted from the buffer used in the platelet experiment in order to avoid aggregation. In spite of these different in vitro conditions we find a parallelism in the spontaneous NE-efflux from platelets on one hand and synaptosomes and heart tissue on the other. Our data also demonstrates the different rate of transmitter turnover in these cell systems; synaptosomes have the most rapid uptake and efflux followed by heart tissue and platelets, in decreasing order.
In earlier experiments we have found that ^{14}C-NE seems to be taken up by the alpha-granules of the platelets and released by exocytosis of the whole granular contents. This is indicated by concomitant release of the alpha-granular protein beta-thromboglobulin in stochiometric proportions. Supernatants from the resuspended platelets also had a growth stimulating effect on smooth muscle cells in culture, indicating a parallel release of the platelet derived growth factor. This spontaneous release appears to be unrelated to aggregation and is not due to cell damage and unspecific leakage of cell constituents (15). It rather seems to reflect a basal process of exocytosis.
We are aware that the measured CPM include not only NE but also the possible

metabolites of NE. As we don't think that this fact influences the main conclusions that can be drawn from this and earlier studies, we have chosen to regard the released CPM as ^{3}H-NE.
Neuronal tissue has a very high capacity for accumulating NE, which was demonstrated by the very rapid uptake curves in the synaptosomes. NE is also very efficiently stored in the granules. After chemical or electrical stimulation, NE is released together with intragranular high molecular weight proteins. As no membrane bound constituents of the vesicle are released, nor the proteins from the cytosol, this seems to favour exocytosis as the cause of NE-release (16,17). Whether or not the spontaneous release of NE is the result of exocytosis has, to our knowledge, never been studied. If non-stimulated exocytosis also takes place in neuronal cells, some general mechanisms regulating granule release may be present in platelets, peripheral nerve terminals and in the central nervous system. Further studies to elucidate these mechanisms are needed.

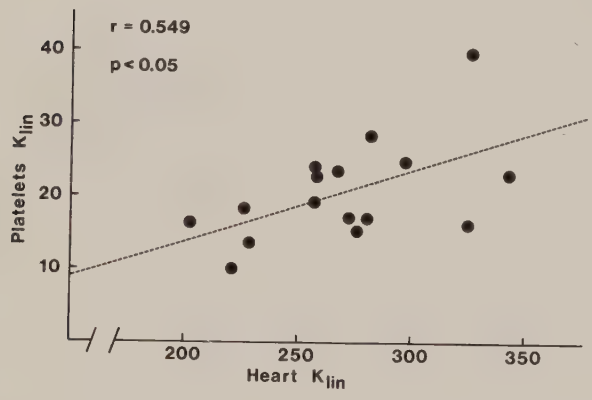

Fig 4.

Correlation between rate constant of norepinephrine efflux, K_{lin} (min^{-1}), from rat platelets and heart tissue (n=16).

This study, together with our earlier findings of platelet release of NE seem to support the hypothesis of increased spontaneous release of NE from sympathetic nerve endings in hypertension. This does not necessarily mean that increased amounts of the transmitter leave the presynaptic neuron, since our model does not account for the effect of presynaptic receptor regulation or capacity of transmitter biosynthesis. Thus, the pathogenetic role of increased spontaneous exocytosis in hypertension needs further evaluation.
Apart from hypertension, our results may also be relevant for research in other disorders, if interest is focused on transmittor release and, more specifically, on exocytosis.

Acknowledgements

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